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<http://dx.doi.org/doi:10.21954/ou.ro.0001021c>

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Glycoproteins and Memory Formation in the Day-Old Chick

By

Kate Koles

A thesis submitted in partial satisfaction of the degree of Doctor of Philosophy.

The Brain and Behaviour Research Group

The Open University, Milton Keynes.

November, 1998

DATE OF SUBMISSION: 27 NOVEMBER 1998
DATE OF AWARD: 15 FEBRUARY 1999

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Acknowledgements

I wish to thank Buca Mileusnic and Steven Rose for their invaluable help and advice throughout this thesis. Also my special thank to Dr Bill McDowell for introducing me to the exciting field of glycobiology and for the opportunity to spend a year at the Glycobiology Unit of GlaxoWellcome, Stevenage. I am grateful to Dr Malcolm Ward for the protein sequencing data and to the members of the Unit for their continuous help, encouragement and entertainment. I would also like to thank the members of the Brain and Behaviour Research Group at the Open University, especially Chris Lancashire and Heather Davies for their helpful guidance and assistance in the lab; and Steve Walters, Dawn Sadler and Karen Evans for looking after the chicks.

Abbreviations

2-AB	2-aminobenzamide
2-dg	2-deoxyglucose
2-dgal	2-deoxygalactose
AAA	Anguilla anguilla
Abs	Antibodies
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP	Alkaline phosphatase
AP-1	Adaptor protein 1
APS	Ammonium persulphate
ATP	Adenosine triphosphate
BCIP/NBT	5-Bromo-4-chloro-3-Indolyl Phosphate/Nitroblue Tetrazolium
BDNF	Brain derived nerve growth factor
BSA	Bovine serum albumin
C18	Carbon18
CaMK	Calcium/calmodulin dependent protein kinase
CD15	Cluster of differentiation 15
CMP	Cytidine monophosphate
CNS	Central nervous system
COPI	Coat protein complex
CRD	Carbohydrate recognition domain
CREB	cAMP responsive element-binding protein
CS	Chondroitin sulphate
DEAE	Diethylaminoethyl
DMSO	Dimethyl sulfoxide
DS	Dermatan sulphate
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra-acetic acid
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
FACS	Flow cytometric analysis
FGF	Fibroblast growth factor
Fuc	Fucose
GA	Golgi apparatus
GAG	Glycosaminoglycan
Gal	Galactose
GalN	Galactosamine
GalNAc	N-acetylgalactosamine
Glc	Glucose
GlcA	Glucuronic acid
GlcN	Glucosamine
GlcNAc	N-acetylglucosamine
GlcNAcT	N-acetylglucosaminyltransferase
GPC	Gel permeation chromatography
GPI	glycosylphosphatidylinositol
HA	Hyaluronic acid
HEPES	2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulphonic acid

HPAEC	High-pH-anion exchange chromatography
HPLC	High-performance liquid chromatography
HS	Heparan sulphate
IdoA	L-iduronic acid
IEG	Immediate early genes
Ig	Immunoglobulin
IMHV	Intermedial medial hyperstriatum ventrale
KS	Keratan sulphate
LPO	Lobus parolfactorius
MALDI-MS	Matrix-assisted laser desorption ionisation mass spectrometry
MAP-1B	Microtubule-associated protein 1B
MeA	Methylantranilate
MOG	Myelin/oligodendrocyte glycoprotein
mRNA	Messenger ribonucleic acid
Mw	Molecular weight
N-CAM	Neural cell adhesion molecule
NeuAc	N-acetyl-neuraminic acid
NeuNAc	N-acetyl-neuraminic acid
NMDA	N-methyl-D-aspartic acid
NMR	Nuclear magnetic resonance
NPGU	Normal-phase glucose unit
O-GlcNAc	O-linked GlcNAc
P4	BioGel P4 gel filtration chromatography (GPC, SEC)
PAGE	Polyacrylamide gel electrophoresis
PAPS	Phosphoadenylyl sulphate
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PED	Pulsed electrochemical detection
PG	Proteoglycan
PKA	cAMP dependent protein kinase A
PKC	Protein kinase C
PSA	Polysialic acid
PS-DVB	Polystyrene-divinylbenzene resin
PVDF	Polyvinylidene difluoride
RAAM	Reagent array analysis method
RER	Rough endoplasmic reticulum
RSD	Retinal spreading depression
SDS	Sodium dodecyl sulphate
SEC	Size exclusion chromatography
Ser	Serine
SGGL	Sulphoglucuronylglycolipid
SPM	Synaptic plasma membrane
TAG-1	Transient axonal glycoprotein 1
TBS	Tris-buffered saline
TFA	Trifluoroacetic acid
Thr	Threonine
TRIS	N-Tris(hydroxymethyl)methylglycine
UDP	Uridine-5'-diphosphate
UEA-I	Ulex europaeus type I

1. Abstract

Passive avoidance training in day-old chicks results in a cascade of biochemical, physiological and morphological events lasting for at least 24 hrs after training. One of these steps is an increase in fucose incorporation into synaptic plasma membrane (SPM) glycoproteins, which also occurs in other animal species and learning tasks. In order to identify these fucosylated glycans, the glycosylation profile of chicken synaptic plasma membrane (SPM) glycoproteins has been investigated.

N- and O-linked glycans were released from delipidated pure SPMs by automated hydrazinolysis and fluorescently labelled with 2-aminobenzamide. Labelled glycans were separated according to their size, charge or hydrophilic profile by gel filtration and high-performance liquid chromatography. In the charged glycan fraction, sialylated, sulphated and phosphorylated glycans were detected, whereas the neutral glycan profile was dominated by oligomannosidic structures. Two of the most abundant complex type oligosaccharides have been sequenced.

The neutral glycan profiles from trained and quiet chicks did not show any difference, however there were indications of possible learning related changes in the charged glycan fraction.

In addition to learning related changes, another analysis investigating the biochemical correlates of neural plasticity using the dark-hatching paradigm was performed. A decrease in sialic acid content in the P2 fraction of dark-hatched chicks was noted, which is tentatively attributed to the undersialylation of small, possibly O-linked glycans.

2. Learning and memory

Learning and memory are very complex phenomena, “one of Nature’s most jealously guarded secrets” (Elbein 1987). A number of scientific disciplines ranging from psychology through molecular biology to quantum physics study different aspects of the same problem. Currently it is believed that the brain (or in a wider concept the nervous system) is the biological substrate of learning and memory, and that the communication between its neurons changes as a result of learning. The mechanisms of these changes are the focus of neurobiology of learning and memory.

Definitions

Although the terms “learning” and “memory” are widely used in our everyday language, a more precise description from a neurobiological perspective feels appropriate.

A basic property of the brain is that its sensory inputs -conveying information from the outside and inside world of an organism- generate a neuronally encoded internal representation of these worlds. *Learning* is defined as the generation or modification of enduring internal representations in an experience-dependent manner and *memory* as the retention of these representations. *Retrieval* is the use of memory in neuronal and behavioural operations (Harland 1994). Learning and memory can also be considered as examples of “neural plasticity”, the term used to describe the ability of the nervous system to change structure and function in response to experience, injury, ageing, stress, hormones, trophic factors or pathological conditions.

Levels of analysis

Inevitably, learning and memory are being studied at several different *levels* of complexity. At the most complex level is the *behavioural* analysis, which is concerned with the overall behaviour of the organisms, and assumes that the rules governing human (or animal) behaviour can be deduced from behavioural observations provided proper psychological and behavioural tests are used.

The next level is at the *organ* level; such studies focus on the overall activity of the brain, which is thought to be generated by the activity of the composite neuronal systems. How the individual neuronal systems work however has to be analysed at sub-organ level, also called the analysis of neuronal circuits. The individual components of these circuits, the neurons and other cellular components of the brain are the subject of *cellular* neurobiology. And finally, the individual building blocks of these cells are studied at the *molecular* level.

With the current trends in the neurobiology of learning and memory the range of investigations is not expected to end at the molecular level. Several hypotheses have been put forward during the past decade about the possible existence of subatomic phenomena in the nervous system, which can be accounted for only from a quantum-theoretical viewpoint. These quantum phenomena are thought to underlie consciousness and even memory formation (Woelf 1998). I point this out, because the core aspects of these theories are based on the presence of carbohydrate structures at the synaptic junction and neuronal cell surface, the primary focus of this thesis (Jibu et al. 1996).

Methodology

A *scientific* approach to learning and memory requires special experimental paradigms, which enable the reproducible quantification of learning and memory. These experimental tools are unique to the neurobiology of learning and memory.

The first such paradigm was introduced by Ivan Petrovich Pavlov, working on the digestive reflexes (salivation) of dogs in Russia at the beginning of the 20th century. The paradigm consisted of repeated exposure of hungry dogs to food preceded by a short light or sound stimuli (conditioned stimuli, CS). After a number of trials the dogs learned that light or sound stimuli was a reliable predictor of food (in other words, the dogs learned to associate light/sound with food). This was measured as increased salivation in response to CS. This paradigm was the first example of an associative learning task (classical

conditioning). There are many variants of the task today, their description can be found in Dudai (1989). Other animal systems and learning tasks have been gradually developed, the best-known and widely used examples include the delayed spatial response task in monkeys, eyeblink conditioning paradigm in rabbits, spatial delayed nonmatching-to-sample task in dogs, brightness discrimination and avoidance conditioning in cats, a large number of spatial tasks in rat, radial arm maze and active avoidance tasks in mice, passive avoidance and imprinting in chickens etc.

Classification of learning and memory

As the number of experimental paradigms and behavioural observations increased, certain common features emerged. These led to the classification of learning and memory into two basic categories, explicit and implicit memories (Squire 1992). Conscious knowledge about facts, people, places and things are examples of declarative memories (particularly well developed in the vertebrate brain), whereas unconscious acquisition of motor skills and other tasks are known as implicit memories. Examples of this latter form of learning include simple associative and (classical conditioning) and nonassociative (sensitisation and habituation) learning.

These two types of learning seem to depend on different neuronal substrates, as lesion studies from humans and later from other mammals indicated that the acquisition of explicit -but not implicit- memories requires the temporal lobe and diencephalic structures (e.g. hippocampus, subiculum and entorhinal cortex). Implicit memories are dependent on the same sensory, motor and associational pathways that are involved in the expression of the learning process (and are most readily studied in invertebrates with relatively simple nervous systems).

Perhaps one of the most obvious classifications takes into account the temporal properties of memory formation, hence the short-, intermediate- and long-term memories. Different biochemical and molecular mechanisms seem to subserve these three types of memories

which exist in parallel, as have been illustrated by genetic and pharmacological studies first in *Drosophila* and later in other organisms as well (DeZazzo and Tully 1995).

In parallel with behavioural neuroscience, the foundations of cellular neurobiology have also been established. Eugenio Tanzi and Ramon Cajal were the first to suggest independently at the turn of the 20th century that the brain is composed of individual cells just like other tissues, and that they are linked together via specific junctions, which later Sherrington called “synapses” (Rozenzweig 1996). The notion, that learning and memory involves alterations in the connectivity between individual neurons can be traced back to these authors. The first significant hypothesis about the possible mechanisms of these changes was published by Donald Hebb in 1940. He suggested, that: “When an axon of cell A is near enough to excite cell B or repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A’s efficiency, as one the cells firing B, is increased” (Hebb 1949). Although he could not tell whether his hypothesis (also called Hebb’s rule) was correct at the time when he published his work, several experiments have now confirmed that Hebbian learning does occur in the nervous system, and is one of the molecular mechanisms underlying certain forms of learning.

A physiological phenomenon, called *long-term potentiation* (LTP), discovered in the rabbit dentate gyrus and first described in detail by Bliss and Lomo (1973) has several interesting properties that make it a candidate mechanism of learning and memory (Baudry et al. 1993).

LTP is a dramatic increase in synaptic efficacy in response to short, high frequency (tetanic) stimulation of monosynaptic glutamergic pathways (Bliss and Collingridge 1993). This increased potentiation can last for several days or longer in vivo, depending on the induction paradigm and the previous history of the stimulated pathway. Moreover LTP is a

Hebbian process in its requirement for coincident activity at pre- and postsynaptic sites during induction.

During LTP induction there is an increase in postsynaptic calcium concentration, however the nature of changes that cause the strength of potentiated pre- and postsynaptic connection to be altered is still unclear. Several components are thought to be involved. An action potential arriving to the presynaptic terminal leads to calcium influx through voltage sensitive calcium channels, which then causes the quantal release of neurotransmitters stored in synaptic vesicles. There are several release sites at presynaptic terminals, however not all of them are released when an action potential arrives, only a fraction (called quantum) is released in a probabilistic fashion. The released glutamate binds to the NMDA receptors, however this binding is not sufficient to induce LTP, because under normal conditions Mg^{2+} blocks the NMDA receptor mediated calcium influx. A brief depolarisation of the postsynaptic site is required to remove the Mg^{2+} blockade, which then leads to increased calcium influx. The ligand- and voltage-dependent characteristics of the NMDA channel provide LTP with Hebbian properties. The increased calcium influx initiates a cascade of biochemical events, including second messenger systems and protein kinases, many of which are now well-understood (Dudai 1989). Therefore the possible mechanism of LTP may involve the alteration of release probability from the *presynaptic* site, the increase in the number of presynaptic release sites or the change in the *postsynaptic* response via modulating the neurotransmitter receptors.

Several different forms of LTP have been described to date, classified into two basic categories, as NMDA receptor and voltage sensitive calcium channel-dependent LTP. It is expected, that the different forms of LTP may use different mechanisms, e.g. maintenance of hippocampal CA1 LTP (an NMDA dependent form of LTP) is thought to be a postsynaptic process requiring a PKA dependent AMPA receptor synthesis (Nayak et al. 1998, Grover 1998). Mossy fibre LTP on the other hand does not require postsynaptic

NMDA receptors for induction, however it depends on the presynaptic calcium-dependent activation of adenylyl cyclase (Salin et al. 1996).

An immense number of studies have focused on LTP as a candidate mechanism of learning and memory. Although it is still controversial whether LTP equals memory, the model, mostly because of its easy experimental manipulation compared to other model systems, has proved to be extremely useful concerning the biochemical and molecular details of short- or long-term forms of synaptic plasticity. The biochemistry of initiation, expression and maintenance of LTP are now well understood, however I will have to restrict my further discussion to the long-term processes, with special focus on the role of **cell adhesion molecules** in the maintenance of LTP.

The long-term maintenance phase of LTP, just like long-term memory formation, requires gene expression and *de novo* protein synthesis (Stanton and Sarvey 1984). Several attempts have been made to isolate and characterise the RNA transcripts and/or their products induced by learning or LTP. Two-dimensional PAGE and recently a much more powerful, subtractive hybridisation strategy has identified a large number of gene products tentatively associated with the processes of synaptic plasticity. In their estimate, Lanahan and Worley (1998) suggest the number of neural plasticity induced genes to be around 30-40 out of which 10-15 are transcription factors. Several of these gene products have been sequenced, perhaps the most astonishing finding was the wide functional variety of proteins induced: enzymes, targeting proteins, cytoskeletal proteins, molecules involved in signal transduction, and a large number of novel, as yet unknown gene products. It is only a question of time, that all these gene products will be sequenced.

Some insight about the possible identity of these proteins was obtained using antibodies against the cell adhesion molecules (CAMs). CAMs are present in the cell membranes and in the extracellular matrix. They are involved in morphogenesis, in cell-cell and cell-matrix adhesion, and in addition to their structural function, also in cellular signalling events. The

majority of cell adhesion molecules (CAMs) is grouped into one of the following families: the cadherin superfamily, the integrins and the immunoglobulin superfamily. CAMs bind either in a homophilic or heterophilic manner. **Cadherins** are calcium dependent, i.e. they require Ca^{2+} for interaction, and they mediate cell interaction via homophilic adhesion. They are present in every tissue type, and N-cadherin is the major component of the postsynaptic densities (PSDs), suggesting that it may be involved in the stabilisation of synaptic structures (Beesley et al. 1995).

Integrins are the primary mediators of cell-extracellular matrix interactions. The binding activity of many integrins depends on divalent cations. Integrins, like cadherins, also interact with cytoskeletal actin proteins talin, vinculin and perhaps other cytoskeleton associated proteins. Integrins are divided into subfamilies, each with a common β -subunit capable of associating with a specific group of α -subunits. The role of integrins in cellular signalling is one of the best-understood among the cell adhesion molecules (Jones 1996, Schwartz et al. 1995).

The **immunoglobulin superfamily** of cell adhesion molecules is characterised by the presence of several immunoglobulin (Ig) domains in the molecule. In addition to the Ig, a variable number of fibronectin domains are also present. Binding typically occurs in a Ca^{2+} -independent and homophilic manner, however certain Ig-like CAMs are capable of heterophilic interaction (N-CAM and L1 binding to neurocan, ICAM to integrins etc.). The cytoplasmic domain of these molecules varies in length and structure as a result of alternative splicing. The prototype of Ig-like CAMs, N-CAM (neural cell adhesion molecule) was isolated in Gerald Edelman's lab and is one of the best characterised and understood adhesion molecules in the nervous system (Hampel et al. 1996, Chothia and Jones 1997).

Returning to LTP, morphological changes have been repeatedly described after LTP induction (Desmond and Levy 1986, Chang and Greengough 1984). As all the three major

types of cell adhesion molecules (integrins, cadherins and the IgCAMs) are present in the cell membranes, including the synaptic plasma membranes, it is anticipated that long-term changes would involve the recruitment of a large number of membrane components. The first evidence for the requirement of a cell-adhesion molecule in LTP was obtained using antibodies against N-CAM and L1. In hippocampal slices from rat brains Luthi et al. (1994) described an impaired development of LTP after antibody application, while the induction or maintenance were not affected. In addition, their studies indicated a requirement for the N-CAM-L1 complex to be formed, as oligomannosidic glycans, which mediate this interaction, had similar effects on LTP as the antibodies against the individual CAMs. Perhaps adding to the evidence supporting a link between LTP and memory are results reporting on the amnesic effects of anti-N-CAM and anti L1 antibodies in different animals and different learning tasks. Doyle et al. (1992b) using the passive avoidance paradigm in rats found the 6-8 hours post-training period to be sensitive to antibody administration. Similarly, the *acquisition* of Morris water maze task was attenuated by anti-NCAM (though not antiL1) antibodies, whereas both antibodies had an effect on *retention* (Arami et al. 1996). In the chick memory formation for the one-trial passive avoidance task was also sensitive to antibodies or peptide fragments of these two cell adhesion molecules, as will be described later.

Evidence for integrins and especially cadherins has been much sparser than for the Ig family of CAMs. The first indications about integrins came from Gary Lynch's laboratory. They found that the RGD peptide involved in integrin-extracellular matrix interactions (Bahr et al. 1997) or antagonists for integrin binding sites (Xiao et al. 1991) interfered with LTP maintenance.

Cadherins, by virtue of their requirement for calcium ions during homophilic binding may act as sensors of extracellular calcium, and hence translate synaptic activity to structural changes at synaptic regions. This has been elegantly demonstrated by Tang et al. (1998),

using antibodies directed against the extracellular domains of N- and E-cadherin (neural and epithelial cadherin, respectively). Treatment of hippocampal slices with these antibodies prior to LTP induction significantly reduced LTP. Because cadherins are present as calcium-dependent homophilic dimers, prior disruption of these dimers is required for antibody recognition. This is thought to be achieved by transient changes in calcium concentration during LTP induction, because artificially high calcium concentrations during LTP induction prevented the action of the above mentioned antibodies.

2.1 Animal models and learning tasks

As mentioned previously, many experiments on learning and memory are based on animal model systems in combination with special learning tasks. The advantages of these model systems are the reproducibility of learning and the possibility of multidisciplinary investigation within the same experimental organism using the same learning paradigm. Moreover, as the human (or other mammalian) brains have an extremely complex structure, less elaborate nervous systems are more amenable to analysis (especially when interventive approaches are required). There is an inherent –although not often articulated– evolutionary assumption behind the choice of these methods, treating nonhuman brains as evolutionary ancestors of the human brain. It is still controversial, to what extent these generalisations and extrapolations from one system to another might be valid (Preuss 1995). Nevertheless the basic principles of synaptic transmission are the same in virtually every nervous system studied, therefore the use of model systems certainly contributes to a better understanding of the nervous system in general.

Pavlov's classical conditioning paradigm was the first experimental system used for the study of learning and memory, since then however many other experimental paradigms and animal models have been developed. Kandel's group made use of the large, easily accessible neurons of *Aplysia*, a sea-slug with simple, well understood nervous system.

Habituation and sensitisation, two examples of implicit memories can be readily studied in this animal. Another well-known model system is the conditioning to phototaxis in *Hermissenda crassicornis*, another sea snail. Among the invertebrate model systems the fruit-fly *Drosophila* has been widely used, especially for the analysis of the genetic basis of learning and memory. Imprinting, food-storing and song-learning are other behaviours that are commonly investigated in birds, like chickens, scrub jays and zebra-finches, res. Examples of mammalian systems are the mouse, rat, rabbit, dog and monkey with a large number of different tasks focusing on different types of memories, ageing or neurodegenerative diseases. It would be outside the scope of this thesis to describe these animal systems in detail, therefore I will restrict my discussion to the passive avoidance learning paradigm in the day-old chicken.

2.2 Learning and memory in chickens- the passive avoidance learning task

One of the animal model systems used with great success during the past decade is the one-trial passive avoidance task in day-old chickens. The task was introduced by Cherkin and is based on the observation that day-old chickens spontaneously peck at bright objects in their environment (Cherkin 1969). If chicks are presented with a bead, which is coated in an aversive solution, they peck at it and then show a characteristic disgust response. On testing sessions for at least 24 hours following training above 70% of the chicks avoid a similar but dry bead. In contrast, chicks that were trained on a bead dipped in water continue to peck at the dry bead after training. Thus chicks are able to learn to associate a particular bead with an unpleasant experience. This is the basis of the one-trial passive avoidance learning paradigm.

The chicken model system has a number of advantages: chicks are easy and simple to keep in large numbers without substantial financial impact. The one-trial passive avoidance paradigm –as its name implies- is acquired after a single training session, therefore precise temporal measurements are also possible. Moreover day-old chicks have a reduced blood-

brain barrier, enabling the easy diffusion of pharmaceutical or other agents (like precursors, antibodies etc.) in the brain. The incompletely ossified thin skull makes the injections simple, without any obvious effect on the behaviour of the chickens. The effect of drugs or antibodies is monitored during the testing sessions. If the chicks avoid the bead for 24 hours that indicates that the processes of memory formation were not affected by drug treatment, however if they peck at the bead, i.e. they are amnesic, it may suggest a specific role for the target molecule. Further experiments are usually performed to rule out possible non-specific effects of the drug or antibodies (Rose 1981).

The disadvantages of using day-old chickens are due to the rapid development of the brain, with the most complex changes taking place after birth. It is often not simple and straightforward to detect learning-related changes when superimposed on a rapidly developing biochemical background. There can be variations between individuals as well, and to detect small biochemical changes, which fall below the limit of population variability (which is about 10-30%) is impossible. Moreover, the neurochemical mapping of chicken brain is still incomplete.

2.3 Biochemistry of memory

The molecular and biochemical events triggered by the passive avoidance learning task are fairly well understood. It is now widely accepted that the process of long-term memory formation for this task consists of at least two temporally distinct phases. They differ in the underlying biochemical processes as will be described in the following sections.

Short-term processes

Immediately after pecking at the MeA bead, there are detectable changes in calcium influx, measured by altered calcium influx into synaptosomes. This increase in calcium influx in the MeA trained group has been attributed to voltage sensitive calcium channels, as it was observed after 70 mM KCl stimulation, and is confined to 0-5 minutes after training (Salinska et al. 1998). The increased calcium influx triggers the increased release of

neurotransmitters, including glutamate (Daisley and Rose 1994). This transmitter acts through the NMDA type and non-NMDA glutamate channels, since antagonists for these channels cause amnesia if injected 10 or 25 minutes after training (Rickard et al. 1994). With a similar time course the binding activity of NMDA receptors increases in the left IMHV, but declines to control values within 3 hours (Stewart et al. 1992). Another line of evidence comes from measurements of calcium influx into synaptosomes upon incubation with 0.5 mM NMDA. At this concentration there is a significant increase in calcium influx in trained chicks at 10-30 minutes after training (Salinska et al. 1998).

Retrograde messengers (released from the postsynaptic terminal and acting on the presynaptic site, [Medina and Izquierdo 1995]) have been recently identified to have an important role during the processes of memory formation. Holscher and Rose (1992) injected nitroarginine, an inhibitor of nitric oxide (NO, a retrograde messenger) synthesis 1 hour before training, and those chicks were amnesic for the task when tested as early as 30 minutes after training. Enhanced arachidonic acid release (another molecule thought to be involved in the retrograde communication between the post- and presynaptic site, Medina and Izquierdo [1995]) have also been observed after training (Clements and Rose 1996). Pretraining injections of phospholipase A2 (the key enzyme in arachidonic acid release) and lipoxygenase inhibitor, nordihydroguaiaretic acid (NDGA), cause amnesia as well (Holscher and Rose 1994). However the mechanisms of amnesic action in the case of NDGA are not well understood.

Other short-term changes identified in the chick include the presynaptic phosphorylation of B-50 (or GAP-43) (Bullock et al. 1990). B-50 is a protein kinase C (PKC) substrate and Zhao et al. (1995a) described an increase in PKC activity 30 min. after training. Also, inhibitors of PKC, such as mellitin, H-7, H-8, H-9 have been shown to prevent memory consolidation (Ali et al. 1988). The distribution of the enzyme changes as well, 30 minutes

after training there is a small, but significant increase in the proportion of SPM bound PKC in the left IMHV (Burchuladze et al. 1990).

An increase in cAMP levels 30-60 minutes after training has been reported by Brown (1984). That this rise in cAMP levels may be required for the activation of another kinase, the cAMP dependent protein kinase, PKA was suggested by Zhao (et al. 1995b), who showed inhibition of memory consolidation for the one-trial passive avoidance task using specific PKA inhibitors. Ca^{2+} /calmodulin dependent protein kinase (CaMK) has also been implicated by the same authors on the basis of its increased activity within 10 minutes after training lasting up to 70 minutes after training, as well as on the basis of sensitivity to specific inhibitors (KN-62).

Long-term processes

The above mentioned protein kinases by phosphorylating target molecules most probably contribute not only to the short- and intermediate-term phases of memory via altering the biophysical properties of the neurotransmitter receptors and/or ion channels; but also to the long-term phases by triggering specific biochemical signalling cascades leading to gene transcription (Bailey et al. 1996). One possible mechanism is by modulating specific transcription factors (like CREB, Silva et al. 1998). Activation of these factors leads to the expression of a specific class of genes, the immediate early genes (IEGs). IEGs code for transcription factors, which are required for the transcription of another set of genes, coding for enzymes, cell adhesion or other molecules required for the restructuring of existing or the growth of new synapses.

Two of the IEGs have been identified in the chick, they are the *c-fos* and *c-jun* proto-oncogenes, and an increase in *c-fos* mRNA expression 30 minutes after training has been reported by Anokhin et al. (1991). However, as *c-fos* expression is very sensitive to a variety of sensory stimuli, another early learning task in the chick has been designed which was able to discriminate between learning and non-learning related gene induction. In

those experiments the greatest increase in *c-jun* expression occurred in the group which had to *learn* certain aspects of the task (Anokhin and Rose 1991).

The initiation of transcription by the members of the Fos and Jun family of transcription factors is very complex and requires the assembly of a multimeric protein complex (activator protein AP-1), which then binds to the AP-1 recognition motif of the promoter.

It is unknown, which genes are induced as a result of IEG activation in the chicken, but *de novo* protein synthesis is necessary if long-term memories are to be formed. This is perhaps one of the earliest observations relating to the biochemistry of memory. Antibiotics, like cycloheximide, puromycin and anisomycin, are inhibitors of protein synthesis and if administered to animals around the time of training they cause amnesia which is apparent from approximately 1 hour after training, depending on the learning task (Flexner and Flexner 1966, Squires and Barondes 1972). Administration of these drugs is not effective continuously beyond 1 hour, they inhibit memory formation only at certain time windows. The possibility of temporally restricted “waves” of protein synthesis has been examined in the chick by Freeman et al. (1995). Anisomycin was injected at 13 time points before or after training, and two critical time-windows identified, when drug administration resulted in profound amnesia. The first wave occurs at 0.5-1.5 hours after training, the second between 4 and 5 hours after training.

Two lines of evidence suggest that glycosylation of these newly synthesised glycoproteins is also required for memory formation. The first line of evidence has been obtained using radiolabeled fucose injections into the brain before training on the task. An increased incorporation of this glycoprotein precursor into synaptic plasma membrane fraction was shown in trained chicks (Sukumar et al. 1980, Burgoyne and Rose 1980). This increase in fucose incorporation is specific to the processes of memory formation and not simply a consequence of the training procedure, because a brief, subconvulsive transcranial electric shock immediately after training renders chicks amnesic for the task and those chicks do

not show an increase in fucose incorporation. However, when the electric shock is delayed to 10 minutes after training, no amnesia is induced, and the increase in fucose incorporation is also present (Rose and Harding 1984). Thus experience of the training paradigm without memory formation does not lead to increased fucose incorporation.

Two enzymes are critical for the incorporation of fucose into glycoproteins, phosphorylation of L-fucose by a fucokinase leads to the activation of free fucose and an UDP-fucosyltransferase transfers the fucose to an acceptor. The activity of fucokinase but not of fucosyltransferase, changes after training, suggesting that the altered fucokinase activity may account for the increased fucose incorporation mentioned above.

The second line of evidence came from studies using a specific deoxysugar, 2-deoxygalactose (2-dgal), which competes with galactose, however a missing hydroxyl residue on the C2 carbon prevents the attachment of fucose. This leads to the reduction of fucose incorporation in 2-dgal treated samples. If fucosylation is necessary for memory formation then 2-dgal treatment –and hence a reduction in fucose incorporation- should prevent memory formation. Jork et al. (1986) have used this sugar analogue for the first time in rats, they found both an amnesia for the brightness discrimination task and a reduction in fucose incorporation into a number of synaptic glycoproteins (Jork et al. 1989). In the chick 2-dgal injections at different time points after training have identified two time windows of sensitivity to this drug (Scholey et al. 1993, Crowe et al. 1994). The first time window (40 minutes after training) precedes the onset of amnesia induced by protein synthesis inhibitors. The second time window however sets in after the anisomycin sensitive window. An explanation of these findings was offered by Rose (1996), who suggested that fucosylation of the already existing proteins may take place during the early phases (0.5-1 hour after training), whereas during the second time window the newly synthesised proteins are being posttranslationally modified.

The identity of these fucosylated glycoproteins is mostly unknown. It was reasoned that if long-term memory formation requires the formation of new or restructuring of existing synapses, then structural molecules, like cell-adhesion molecules, might be involved. Indeed, antibodies against N-CAM and L1 caused amnesia for the passive avoidance learning task. These two cell adhesion molecules of the immunoglobulin superfamily may have different roles in the consolidation process, because antibodies against L1 were amnesic if injected either 30 minutes pretraining or 5.5-8 hours after training, whereas N-CAM antibodies were amnesic only during the second time window (Scholey et al. 1995). Radiolabeled fucose incorporation in combination with polyacrylamide gel electrophoresis of the synaptic plasma membrane glycoproteins has also been used to identify the glycoproteins, which show altered fucose incorporation after training (Bullock et al. 1992). A range of glycoprotein bands at 6 and 24 hours after training was identified in the lobus parolfactorius (LPOs) with altered (decreased or increased) fucose incorporation. However the identity of these glycoproteins is unknown.

An important function for fucose α (1-2)galactose-containing glycoproteins is suggested by the above 2-dgal studies, because if the synthesis of these structures is prevented by 2-dgal, long-term memory formation is impaired. The identity of these glycoproteins is not yet known in the chick, however recently in the rat brain cadherin, the NR1 subunit of the NMDA receptor and a SPM glycoprotein "gp65" have been found to be immunoreactive with mono- and polyclonal antibodies raised against the Fuc α 1-2Gal epitope (Smalla et al. 1998). The glycan chains recognised by these antibodies are currently being characterised (Dr. Smalla, personal comm.). Analysis of the total neutral N-linked glycan pool from adult rat brains (Chen et al. 1998) did not detect α 1-2fucosylated glycan structures, indicating that these structures are O-linked, negatively charged or that they are minor components of the total N-linked glycan pool.

Neurotrophins, glucocorticoids and memory

Neurotrophins and glucocorticoids are two classes of modulatory molecules that were found to have significant effects on neural plasticity, including learning and memory (Thoenen 1995, Smith et al. 1995, McEwen and Sapolsky 1995). Glucocorticoids are released during stressful situations and modulate learning positively or negatively, depending on the amount of glucocorticoids released. Sandi and Rose (1994a) using specific glucocorticoid receptor antagonists were able to prevent long-term memory formation in the day-old chicks for the strong* version of the passive avoidance training paradigm. Intracerebral corticosterone injections on the other hand, for up to 1 hours after training on the weak version of the task, enhanced memory retention for the task for at least 24 hours (Sandi and Rose 1994b) further confirming the role of glucocorticoids in the processes of long-term memory formation. The actions of this glucocorticoid are possibly mediated via protein synthesis, including fucosylated glycoproteins (Sandi and Rose 1997).

Neurotrophins are also required during the consolidation phase of the biochemical cascade, as antibodies against the brain-derived neurotrophic factor (BDNF) cause amnesia within 3 hours after training if injected around the time of training. Their effect seems to involve the modulation of presynaptic proteins SNAP-25 and syntaxin in the left IMHV, as a decrease in these proteins was found in the anti-BDNF treated animals (Johnston et al. 1998).

2.4 Identification of the brain regions involved in passive avoidance learning

Sokoloff introduced a method suitable for monitoring the biochemical activity of specific brain regions using a radiolabeled glucose analogue (Sokoloff et al. 1977). Glucose is

* During the strong training paradigm the bead is dipped in 100% methylantranilate, and memories for this training last for at least 24 hours after training. The weak version of the task uses only 10% methylantranilate during training and the memory for the weak task lasts only for several hours.

taken up by nerve cells depending on their energetic demands. More active cells would take up more glucose. If a modified and radiolabeled analogue of glucose, 2-deoxyglucose (2-dg), -which cannot be metabolised by the cells-is used, it will be taken up and accumulate in the cells without further processing. Subsequent autoradiography of brain slices will detect the regions with the highest glucose uptake. When chicks were given a 30 min pulse of 2-dg just prior to training with the MeA bead, the trained chicks showed a greater accumulation of 2-dg in the intermediate medial hyperstriatum ventrale (IMHV), lobus parolfactorius (LPO) and paleostriatum augmentum (PA), with the greatest changes occurring in the IMHV and LPO of the left hemisphere (Kossut and Rose 1984, Rose and Csillag 1985). Lesion studies further confirmed these sites and extended our knowledge with their temporal and sequential involvement in this process (Rose 1991). Lesions to the left IMHV on the day of hatching caused amnesia 3 hours after training on the next day. Right IMHV or posttraining IMHV lesions had no effect. This suggests that the left IMHV is necessary only for the acquisition but not for storage of the memory. LPO lesions after training disrupt memory for the task. However, LPO is not the definite and only storage site of memory, as pretraining lesions of LPO had no effect on acquisition or retention. According to these results, the memory trace seems to “flow” from the left through the right IMHV to the LPO. Pretraining right IMHV lesions abolished the amnestic effect of posttraining LPO lesions, because the memory trace could not get to the LPO from the left through the right IMHV. These data illustrate that memories are temporally and spatially distributed and not localised to a single region.

2.5 Morphological changes after passive avoidance training

The notion of morphological changes accompanying a learning experience has often appeared in the earlier literature. These suggestions were based on the hypothesis, that alterations in synaptic connectivity are required for long-term memory formation.

Changes in synaptic parameters after passive avoidance learning in the chick have been reported by Stewart et al. (1984). In the medium hyperstriatum ventrale (MHV) the hemispheric differences in the number of synaptic vesicles were reversed by training, also the length of the postsynaptic densities decreased in the right MHV. When Golgi-impregnated neurons from the IMHVs were examined, increases in spine density and dendritic branch pattern were noted 24 hours after training in the left and to a lesser degree in the right IMHV. In the LPO region increases in the synaptic density in both hemispheres 24 and 48 hours after training have also been reported (Stewart et al. 1987, Hunter and Stewart 1993). An examination of the spatial organisation of the synaptic components in the LPO at 24 hours after training revealed a shift of synaptic vesicles away from the active zone in the right hemisphere (Rusakov et al. 1993). Surprising morphological changes occurring early after training (i.e. before the window of sensitivity to protein synthesis) were reported by Doubell and Stewart (1993). They have described an approximately 77% increase in the density of axospinous synapses in the right IMHV of the MeA trained birds as compared to water trained animals, and a 57% decrease in the mean projected height of the synapses 1 hour after training. These differences were not apparent 24 hours after training.

2.6 Aims

The previous sections described our current knowledge of the biochemical cascade and the long-term changes associated with memory formation for the one-trial passive avoidance task in the day-old chicken. The model is one of the most thoroughly understood systems in the neurobiology of learning and memory, though many questions still remain to be answered. One of these unexplored areas are the fucosylated cell adhesion molecules. The identity, expression profile, regulation, or glycosylation profile of these glycoproteins is still unknown. Changes in the rate of fucose incorporation in other animals, experimental paradigms or even rearing conditions were often noted (Routtenberg et al. 1974; Pohle et

al. 1979; Morgan and Routtenberg 1979, Popov et al. 1976, Popov et al. 1980, Rose and Harding 1984, McCabe and Rose 1985), therefore this change in glycoprotein mobilisation may be one of the universal mechanisms of neuronal plasticity. The number of studies on the specific role of fucosylated structures in the brain is limited, despite the exciting findings, like the enhancement of LTP by fucosyllactose or fucose administration in the brain (Krug et al. 1994).

Deciphering the identity of the fucosylated *glycan* structures and the glycoproteins carrying these sugars is a prerequisite for understanding the molecular details of synaptic communication and ultimately memory formation.

In this thesis the identity of *glycan* structures, which show altered fucose incorporation after training was explored. Ideally, the glycosylation profile of individual glycoprotein bands that show changes in fucose incorporation should have been compared (Bullock et al. 1992), however this was not possible due to the lack of antibodies against these glycoproteins and because of the generally low yield of *individual* glycoproteins from the chicken brain.

The original observations of the 29%, 16% and 26% increases in fucose incorporation (at 0.5h, 3h and 24h, resp.) as detected in the particulate fraction of anterior forebrain (Sukumar et al. 1980) and a 16% increase in the right forebrain base in *in vitro* slices (McCabe and Rose 1985) prompted me to compare the glycan profiles of the SPM glycoproteins from trained and quiet chickens. It was hypothesised that certain glycan structures would be selectively expressed and/or altered in proportion only in the trained animals. Therefore I expected that comparison of the glycan profiles from trained and control animals would detect these structures, and in addition a general characterisation of the chick SPM glycoprotein glycans would be achieved.

3. General principles of glycosylation

Naturally occurring molecules containing covalently linked carbohydrates constitute the family of glycoconjugates. All animal cells contain glycoconjugates on their surface as well as inside the cell. The composition of these molecules is not constant, it changes with development, ageing, or even *in vitro* in cell lines with the differentiation state of the cells. There are differences between animal species when their corresponding tissue oligosaccharide profiles are compared, i.e. glycosylation is species-specific. Also, within a single animal, different organs and tissue types have distinct glycan profiles (tissue-specific glycosylation). The function of this species- and tissue-specific glycosylation is mostly unknown, although it is suggested that differential expression and/or activity of the glycosyltransferases/glycosidases is one of the underlying mechanisms.

The knowledge of the exact structures of naturally occurring oligosaccharides has expanded during the last few decades, however understanding of their function is far less progressed. This might be due to the lack of experimental systems and methods suitable for examining the role of glycosylation. The equivalent of site specific *protein* mutagenesis for *glycan* structures is not yet available.

Despite their enormous variability, naturally occurring oligosaccharides contain only a limited number of possible monosaccharide constituents. They are the neutral **hexoses** α -D-glucose, α -D-galactose, α -D-mannose; the **deoxyhexose** α -L-fucose; the **hexosamines** N-acetyl-D-glucosamine, N-acetyl-D-galactosamine; the different derivatives of α -N-acetylneuraminic acid; the **pentoses** β -D-xylose, L-arabinose and the **hexuronic acids** D-glucuronic acid and L-iduronic acid. Each monosaccharide can be attached in α - or β -linkage via its C1 position to an underlying acceptor structure. The only exception is sialic acid, which is linked through its C2 (Figure 3.1a). This position is indicated as the first number in the general nomenclature of the oligosaccharides. The second number indicates

the position of the acceptor structure where the attachment occurs, e.g. Fuc α 1-2Gal indicates fucose α -linked via its C1 to the C2 of galactose (Figure 3.1b).

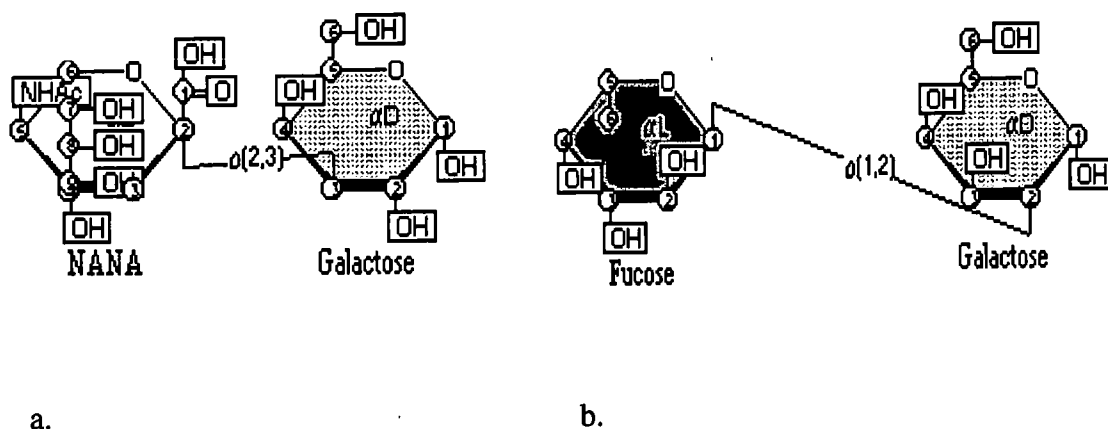


Figure 3.1 a) sialic acid (NANA) linked to galactose in α 2-3; b) fucose linked to galactose in α 1-2 linkage.

Modification of the monosaccharides for instance by phosphorylation, N- or O- sulphation, O-acetylation may also occur, further increasing the variability of the oligosaccharide structures. Glycosyltransferases show very strict substrate specificity, this property is sometimes called the “one enzyme-one linkage” rule, and only two exceptions (an α 1-3fucosyltransferase and a glucosamintransferase involved in O-linked glycan biosynthesis) are known to date. The enormous diversity of the glycan structures therefore requires the action of hundreds of different glycosyltransferases. However, the first few glycosylation reactions are the same for each N-linked oligosaccharide, they share a common core structure, and the specific diversity develops as the glycosylation progresses.

3.1 Biosynthesis of N-glycans

The biosynthesis of N-glycans is initiated by the addition of a precursor sugar, which is attached to dolichol. This precursor is synthesised on the cytosolic side of the rough endoplasmic reticulum (RER) with the attachment of GlcNAc from UDP-GlcNAc to Dol-P. Subsequently, the rest of the precursor becomes added to the molecule step by step to

yield Man5-GlcNAc2-PP-Dol, this metabolic process is called the dolichol-cycle, and is illustrated on Figure 3.2. This structure is transferred to the lumen site of the RER where additional processing occurs until the Glc3-Man9-GlcNAc2-PP-Dol is assembled. The mechanism of the transfer of this bulky hydrophilic molecule through a lipid bilayer is unknown.

An amphomycin* and tunicamycin* sensitive oligosaccharyltransferase (OST) then transfers this precursor to the asparagine residue of the protein, provided this amino acid is surrounded by the N-glycosylation consensus sequence (-Asn-X-Ser/Thr), where X is any amino acid except for proline. The released dolichol-P-P is hydrolysed by a cytosolic phosphatase to yield dolicholphosphate, which then re-enters the dolichol-cycles (see Figure 3.2). This hydrolysis step is sensitive to bacitracin*.

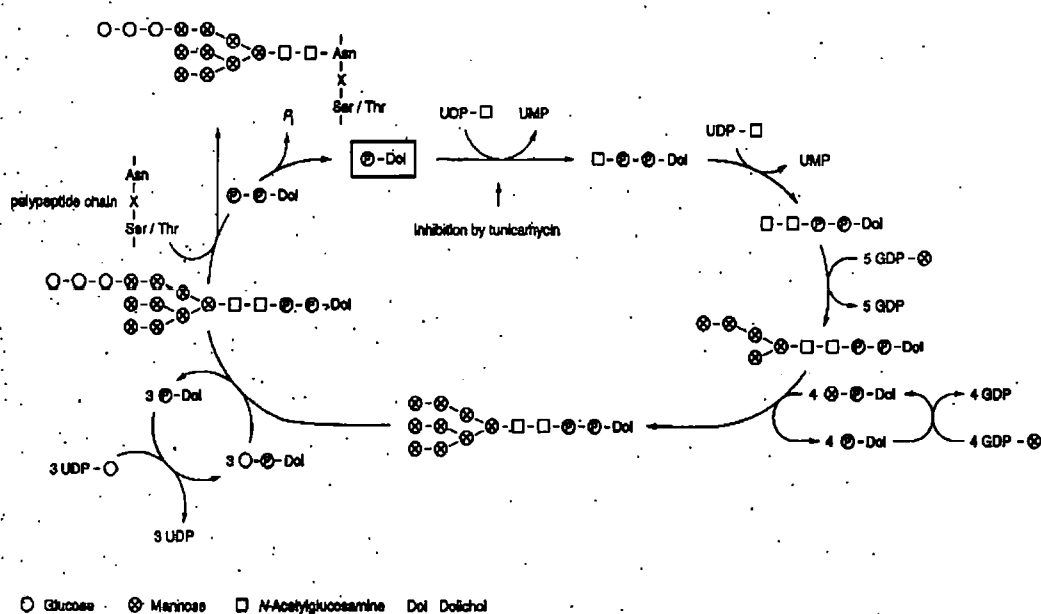


Figure 3.2 The dolichol-pathway of lipid-linked precursor synthesis (from Lehmann 1998).

*Amphomycin is an undecapeptide antibiotic containing either 3-isododecanoic or 3-anteisododecanoic acid attached to an N-terminal aspartic acid in amide linkage. It is thought, that it forms a complex with dolicholphosphate thereby inhibiting further biosynthetic reactions.

*Tunicamycin is a nucleotide based antibiotic originally isolated from *Streptomyces lysosuperificus*. It is a substrate-product transition state analogue of GlcNAc-1-P transferase, and because its very strong interaction with the enzyme irreversibly inhibits this transfer step (Elbein 1987).

*Bacitracin is a polypeptide antibiotic obtained from *Bacillus licheniformis*

Many asparagine residues, despite the correct signal sequence are not glycosylated. It has been proposed that the tertiary conformation of this particular consensus sequence may be critical for the glycosylation reaction to take place. The α -NH of asparagine may form a hydrogen bond with the oxygen of a serine/threonine and the glycosylation enzyme then recognises this particular tertiary structure. The lack of glycosylation at certain sites may be due to the specific tertiary conformation of the protein, which does not allow this special hydrogen-bond to be formed (Shakineshleman 1996).

The oligosaccharyltransferase enzyme complex has been purified from several organisms, including canine, avian, porcine, human and fungal species, and is a complex of three to six subunits. Two of the subunits have been identified as ribophorin I and ribophorin II (Crimaudo 1987). Since the identification of these components, monoclonal antibodies against them have been used to locate N-glycosylation sites in cells, including neurons, where glycosylation has been detected in proximal dendrites as well (Torre and Steward 1996).

Two membrane-bound glucosidases, glucosidase I and glucosidase II, remove the terminal glucose residues. These enzymes are inhibited by 1-deoxynojirimycin and N-methyl-1-deoxynojirimycin*. Improperly folded glycoproteins trimmed by α -glucosidase II are bound by calnexin and/or calreticulin and transiently retained in the ER. They become transiently reglycosylated by the UDP-glucose: glucosyltransferase (Parker et al. 1995). After being properly folded, monoglucosylated glycoproteins may become the substrate for α -glucosidase II and can leave the ER (Bergeron 1997). This sequence of calnexin/calreticulin mediated steps is also called "quality control" of newly synthesised proteins.

*Deoxynojirimycin is a competitive active site directed inhibitor of several different glucosidases found in different tissues.

After the Glc residues have been removed from the properly folded glycoprotein, one Man residue from the Man(α 1-6) branch is cleaved by the ER resident α 1-2-mannosidase. These early steps of processing occur in essentially all organisms, plant and animal, but continued processing, which generates the diverse structures, is characteristic for higher animals (Varki and Freeze 1994).

The glycoprotein with the Man₈-GlcNAc₂ side chain is then transported from the RER to the *cis* cisternae of the Golgi stacks. This transport is thought to be receptor mediated with a receptor recognising the deglycosylated oligosaccharide chain and can be inhibited with brefeldin A.

In the Golgi stacks further processing occurs depending on the final destination of the glycoprotein. These are the lysosomal (Figure 3.3) and nonlysosomal processing pathway. Enzymes targeted to the lysosomes contain at least one conserved recognition domain, which serves as a binding site for phosphotransferases.

Two mannose residues of the **lysosomal enzymes** become phosphorylated in a two step process in the Golgi cisternae (Figure 3.3). Glucophosphorylation by N-acetylglucosaminyl-phosphotransferase is followed by the removal of the capping glucosamine by the action of N-acetylglucosamine-1-phosphodiesterase α -N-acetylglucosaminidase. The exposed mannose-6-phosphate residues then interact with the corresponding receptor in the *trans*-Golgi network. The receptor-enzyme complexes are segregated into nascent clathrin-coated vesicles destined for the early endosomes (Meresse et al. 1993).

N-methyldeoxynojirimycin displays a similar specificity but is more effective in crossing the cell membrane due to an additional N-methyl group, and hence a more potent inhibitor.

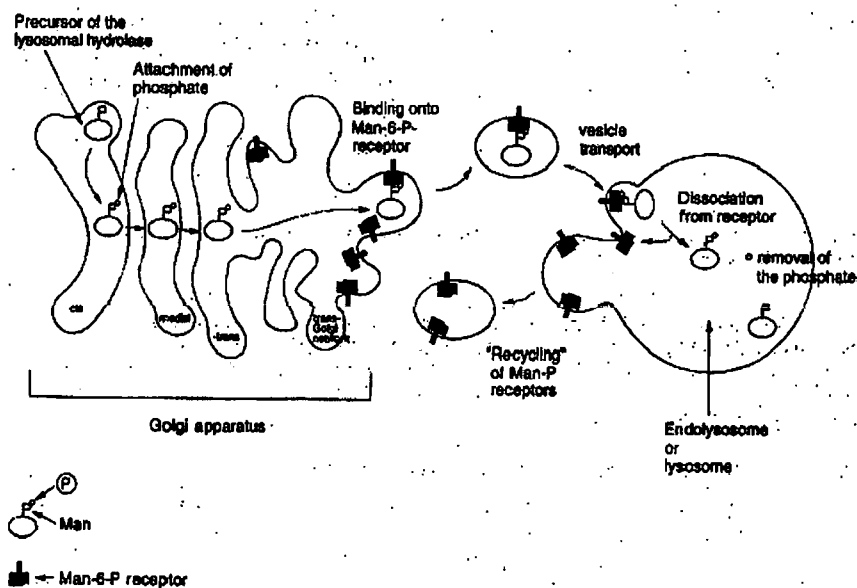


Figure 3.3. The lysosomal pathway of N-linked glycan processing (from Lehmann 1998).

Non-lysosomal proteins are processed through the action of mannosidases, which are the intermediates to different high-mannose structures or precursors to hybrid or complex structures. The unphosphorylated octamannosyl derivative can be a substrate for mannosidase I, which can cleave 3 terminal mannose residues, leaving the pentamannosyl structure as the precursor for hybrid and complex type chains (step iii, Figure 3.4). The critical enzyme for further processing is N-acetylglucosaminyltransferase I (GlcNAcT I) which transfers a single GlcNAc residue in β 1-2 linkage to the α 1-3 linked mannose of the trimannosyl core (step iv, Figure 3.4). This is the most critical step in N-linked glycan processing, as without this step no hybrid or complex type structures can be formed. Indeed, mice lacking this enzyme die at midgestation (Ioffe et al. 1997).

α -mannosidase II is the first enzyme on the **complex** type synthetic pathway (step v' Figure 3.4), it removes 2 mannose residues from the α 1-6 arm of the core. Glucosaminyltransferase II may introduce a β 1-2 linked GlcNAc residue to this arm (step vi). Further processing of this structure through the action of galactosyl-, fucosyl- and sialyltransferases leads to the formation of biantennary complex type chains (steps x, xi). In contrast to GlcNAcT II, GlcNAcT III transfers a GlcNAc in β 1-4 linkage to the core

mannose, generating the so-called bisecting structure (step ix). If this latter enzyme follows the action of mannosidase II, bisecting structures are generated. They can be further processed to tri- and tetraantennary structures by GlcNAcT IV and GlcNAcT V, res. (steps vii and viii).

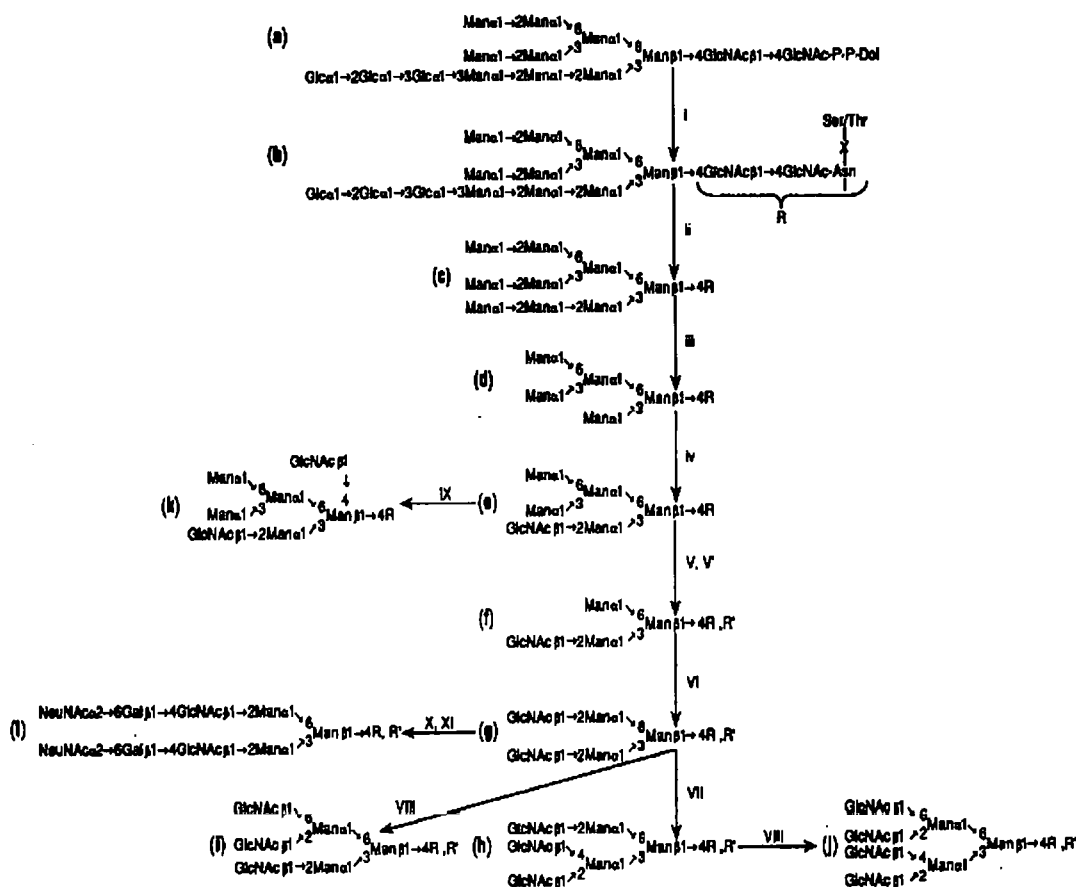


Figure 3.4. The enzymes involved in the early processing steps of N-linked glycan biosynthesis. i- oligosaccharyltransferase; ii-ER glucosidase I and II; iii-ER α -1,2-mannosidase I; iv- N-acetylglucosaminyltransferase I; v- Golgi α -mannosidase II; v'- (Fuc to GlcNAc) α -1,6 fucosyltransferase; vi- N-acetylglucosaminyltransferase II; vii- N-acetylglucosaminyltransferase IV; viii- N-acetylglucosaminyltransferase V, ix- N-acetylglucosaminyltransferase III; x- β 1,4-galactosyltransferase; xi- α -2,6-sialyltransferase; (Kornfeld 1985, Fukuda 1994).

Hybrid type structures are formed when the of α -1-6 arm mannose residues are not cleaved by the action of mannosidase II. The $\text{GlcNAc}\beta 1$ -2 $\text{Man}\alpha 1$ -3 arm of the pentamannosyl structure can be further extended by the action of galactosyl-, fucosyl- and sialyltransferases. If this processing is preceded by the action of GlcNAcT III, bisecting hybrid structures are generated (step ix).

High-mannose structures form when the α 1-3Man arm of the core structure is not modified by GlcNAcT I. As these structures are usually intermediates of partially processed Man9 structures, they contain a variable number (5-9) mannose residues.

Diversity of N-glycans

The above summary deals with the general profile of N-glycans and the biosynthetic steps leading to oligomannosidic, hybrid or complex type N-linked structures. But is it possible to explain the diversity of the naturally occurring glycans? Are there any common principles that could account for this diversity? The Man3GlcNAc2 core pentasaccharide, which is common to all N-linked glycans, can be further extended only in a limited number of ways. Six different structures can emerge, namely the mono-, bi-, tri- (2,4 and 2-6 branched), tetra- and pentantennary glycans (with or without the bisecting GlcNAc). These antennae can be further elongated, with terminal sequences. Therefore it is the combination of the branching (i.e. number of antennae) and the terminal sequences that gives rise to the observed versatility of the complex-type glycans.

According to Fukuda (1994) the terminal sequences can be roughly classified into 5 groups (Figure 3.5). The most common N-acetyllactosamine residue (Gal β 1-4GlcNAc β 1-; group 1) can be found as a monomer, or can be further elongated to form polylactosamine chains.

Group 1	Gal β 1-4GlcNAc β 1-
Group 2	Gal β 1-3GlcNAc β 1- Gal β 1-3GlcNAc β 1- NeuNAc α 2-6Gal β 1-4GlcNAc β 1- NeuNAc α 2-3Gal β 1-4GlcNAc β 1-
Group 3	NeuNAc α 2-3Gal β 1-3[NeuNAc α 2-6]GlcNAc β 1- Gal β 1-4[Fuc α 1-3]GlcNAc β 1- Gal β 1-3[Fuc α 1-4]GlcNAc β 1- NeuNAc α 2-3Gal β 1-4[Fuc α 1-3]GlcNAc β 1- NeuNAc α 2-3Gal β 1-3[Fuc α 1-4]GlcNAc β 1-
Group 4	R-(Gal β 1-4GlcNAc β 1-3) _n Gal β 1-4GlcNAc β 1- (NeuNAc α 2-8) _n NeuNAc α 2-3Gal β 1-4GlcNAc β 1-
Group 5	SO ₄ ⁻ (3 or 4)-GalNAc β 1-4GlcNAc β 1- NeuNAc α 2-3GalNAc β 1-4GlcNAc β 1-

Figure 3.5. Terminal sequences of complex N-linked oligosaccharides according to Fukuda (1994)

The second group is characterised by the terminal monosaccharides linked to galactose or glucosamine. These are the galactose in β 1-3 linkage to GlcNAc, the α 1-3 linked galactose which is linked to another galactose, sialic acids in α 2-3 or α 2-6 linkage to galactose and fucose α 1-2 linked to galactose. Some of these structures can be found in combination, like fucose and sialic acid, they comprise group 3 (see Figure 3.5). Polymers of α 2-8 linked sialic acids and of lactosamine units are in group 4. Sialic acid only forms linear structures, whereas polylactosamine can be linear or branched. Finally, the fifth group contains terminal galactosamine (as opposed to galactose), which can be sulphated or sialylated.

3.2 O-linked or mucin-type oligosaccharides

In addition to N-linked glycans which are linked to the polypeptide through the amide group of asparagine, glycoproteins containing sugars attached through the hydroxyl residue of serine or threonine are called **O-linked** or mucin type glycans. Oligosaccharides

belonging to this group are classified on the basis of the amino acid, to which the sugars are attached (serine or threonine), as well as on the basis of the glycan structure and composition. As the first O-linked glycans were isolated from mucus, they are also called mucin-type structures. Seven different categories of O-linked structures can be described, they are:

GalNAc linked to Ser (Thr) (**mucin-type**)

GlcNAc linked to Ser (Thr) (**O-GlcNAc type**)

Xylose linked to Ser (Thr) (**as in proteoglycans**)

Galactose linked to hydroxylysine

Xylosyl glucose or glucose linked to Ser (Thr)

Fucose linked to Ser (Thr) (**O-fucosylation**)

Among the O-linked oligosaccharides those containing N-acetyl-galactosamine at the core are the most abundant. This type of glycosylation is initiated by one of the polypeptide GalNAc-transferases probably in cis-Golgi. They constitute a family of at least 8 enzymes, which are differentially expressed and have both common and distinct (Hansen et al. 1997). An interesting property of one of these enzymes (GalNAcT I) is that it contains 3 potential N-glycosylation sites. At least one of these sites has to be occupied in order for the enzyme to exit the ER (Piller 1997).

No simple consensus-like rule can be deduced for O-glycosylation from the protein sequences analysed so far (Hansen et al. 1997), though the acceptor sequence context seems to be different for threonine and serine, and may reflect the existence of several GalNAc-transferases with different substrate specificities.

O-glycosylation sites have been found to cluster and have a high abundance in the N-terminal part of the protein. The structures of O-linked oligosaccharides are heterogeneous, however several common **core** structures can be recognised. They are listed below.

Core I	GalNAc α 1-Ser (Thr)
Core II	GlcNAc β 1-6 <div style="text-align: center;">\</div> Gal β 1-3GalNAc α 1-Ser (Thr)
Core III	GlcNAc β 1-3GalNAc α 1-Ser (Thr)
Core IV	GlcNAc β 1-6 <div style="text-align: center;">\</div> GlcNAc β 1-3GalNAc α 1-Ser (Thr)
Core V	GalNAc α 1-3GalNAc α 1-Ser (Thr)
Core VI	GlcNAc β 1-6GalNAc α 1-Ser (Thr)

Several different pathways are available for the elongation of O-linked chains (Varki and Freeze 1994), but in general, O-linked glycans are not as elaborate as the N-linked structures. The biosynthesis is often terminated with characteristic peripheral structures, like L-fucose, sialic acid or D-galactose. Many terminal structures can be found in common between sphingolipids and O-linked glycans. An example of the typical O-linked biosynthetic pathway is illustrated on Figure 3.6.

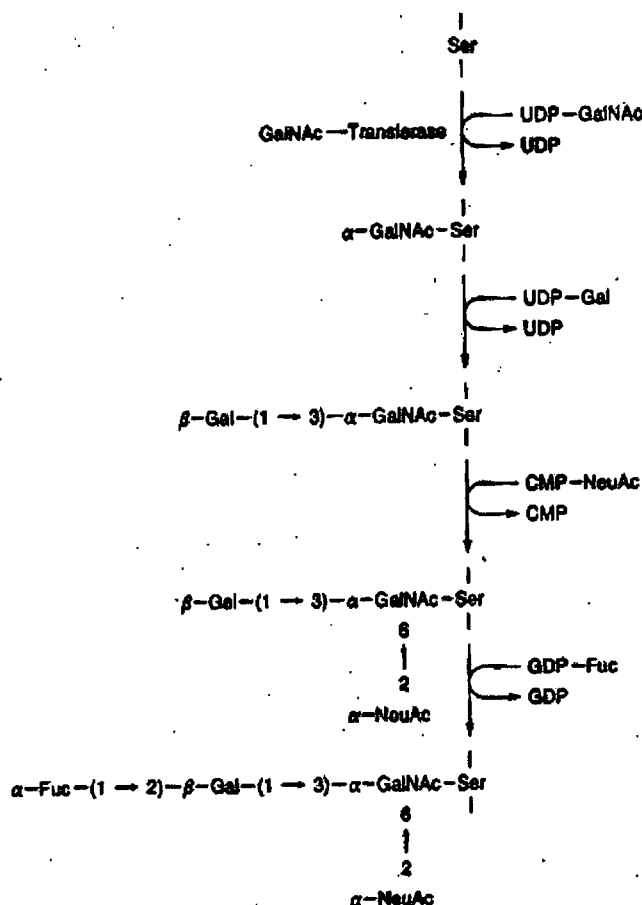


Figure 3.6. An example of the enzymatic steps involved in O-linked glycan processing. Chains are typically not as extended as N-linked complex glycans, but are terminated with sialic acid, fucose, galactose or galactosamine (from Lehmann 1998).

A common property of O-linked glycans is that they tend to occur in patches or clusters on glycoproteins. This may lead to an altered conformation of the glycoprotein itself, adopting a more extended, rod-like structure, which becomes an important factor for membrane glycoproteins/receptors. Moreover, substantial covering of the glycoproteins may protect against the action of proteases, as has been suggested for secreted mucins. In addition, O-linked glycans can serve as ligand for endogenous receptors (Jentoft 1990), and it is often the case, that clustered presentation of ligands results in stronger binding to the receptor.

Novel O-glycosylation pathways have been described recently, including O-linked xylosylglucose, O-linked fucose and a more extensively studied O-linked single GlcNAc. The xylosylglucose-type glycoproteins found to date occur on the epidermal growth factor (EGF)-like domains (Nishimura et al. 1989). Similarly, O-linked fucose was described on

the EGF-like domains of some glycoproteins, however this attachment site is different for the two glycans. O-linked fucose can be even extended with N-acetylglucosamine, galactose and sialic acid. The enzyme responsible for the initiation of this pathway has been recently characterised (Wang et al. 1996) though not yet cloned. The function of this specific glycosylation is controversial at the present.

O-linked single glucosamine is unique amongst the glycoconjugates. O-GlcNAcylation is ubiquitous and abundant on nuclear and cytoskeletal proteins of virtually all eukaryotes. Its turnover rate is much higher than that of the protein backbone to which it is attached. Only a very small percentage of the O-GlcNAcylated glycoproteins have been identified to date, they include transcriptional factors, nuclear pore proteins, cytoskeletal proteins, heat shock proteins, phosphatases, kinases, oncogenes and a subpopulation of estrogen receptors. The O-GlcNAcylation sites of these proteins are similar or identical to their phosphorylation sites. Indeed, O-GlcNAc exists in a dynamic equilibrium with phosphorylation. Therefore three functionally distinct states are possible for O-GlcNAcylated phosphoproteins, naked, phosphorylated or O-GlcNAcylated. Circumstantial evidence suggests, that phosphorylation favours their dissociation, whereas O-GlcNAcylation their association with other proteins. For e.g. the site of O-GlcNAcylation on neurofilament proteins suggests a direct involvement of these sites in protein-protein binding interactions. Tau, a microtubule associated glycoprotein, has also been shown to be heavily O-glycosylated with GlcNAc. In normal neurons tau plays a role in organising microtubules in the axons. However in neurons from Alzheimer's disease patients tau is abnormally hyperphosphorylated, causing it to form abnormal filaments which may be involved in neuronal death (Mandelkow and Mandelkow 1994; Mandelkow and Mandelkow 1993). The findings that the β -amyloid precursor protein is also O-GlcNAcylated and the direct demonstration of altered O-GlcNAc levels in Alzheimer's patients (Griffith et al. 1995;

Griffith and Schmitz 1995) further suggests a putative role for this particular glycosylation in Alzheimer's disease.

A common property of several O-GlcNAcylated proteins (vinculin, talin, synapsin and others) is that they are involved in bridging the proteins/membranes to cytoskeleton (or other proteins) in a phosphorylation dependent reversible manner. Therefore Hart et al. (1995; 1996) suggested, that O-GlcNAcylation might be a mechanism to regulate protein-protein interactions.

3.3 Transporters of nucleotide sugars, nucleotide sulphate and ATP

Oligosaccharides are assembled by the sequential addition of monosaccharides by specific glycosyltransferases in the ER and GA. The monosaccharides have to be in an activated form, i.e. linked to the nucleotide sugar UDP, CMP or GDP. How is the distribution and availability of these sugar precursors regulated? Highly specific nucleotide sugar transporters in the membranes of the ER and Golgi apparatus seem to be involved. These molecules are often organelle specific (Abeijon 1997) and use antiporters with the corresponding nucleoside phosphate as the mechanism to concentrate solutes in the Golgi lumen (Hirschberg 1997). As the supply of nucleotides and nucleotide derivatives is limiting under physiological conditions, reactions with low K_m values may take preference over those with higher ones, thereby the transporters may regulate the nature of glycosylation. However, there are still many open questions about the properties of these molecules. It is not known how are these molecules themselves regulated during different physiological or developmental conditions, how are they targeted to the proper Golgi region, or whether they form functional complexes with glycosyltransferases.

3.4 Topology of the N- and O-linked glycosylation reactions

It has been known for several decades, that the endoplasmic reticulum and the Golgi apparatus are the major sites of glycosylation, including glycoprotein, proteoglycan and

glycolipid biosynthesis. However, the more detailed localisation of the processing enzymes to the RER, ER cis- and trans-Golgi was only possible after the density gradient centrifugation methods became available. Other investigators localised the site of specific oligosaccharide synthesis using lectins. Even finer details and a more definitive localisation was obtained by immunocytochemical methods at light- and electron microscopy level using antibodies against the individual transferases (reviewed by Kornfeld, 1985).

Several questions arise regarding the specific localisation of the glycosyltransferases and glycosidases involved in the biosynthetic reactions. Not only do they have to be transported to their target organelles, but their specific sequence of localisation must be strictly controlled, as the oligosaccharide chain elongation occurs in a sequential manner, the end-product of one enzyme is the substrate of the next enzyme. Often, glycosyltransferases may compete for the same acceptor structures, further illustrating the importance of highly specific enzyme localisation. Despite the obvious importance of these questions, little is known about the mechanisms that target glycosyltransferases and glycosidases to their final destination. All the glycosyltransferases cloned to date share the same type II transmembrane protein topology with a single hydrophobic transmembrane domain, an aminoterminal cytosolic tail and a carboxyterminal luminal domain. The apparent lack of sequence homology excludes the possibility of a signal sequence responsible for the subcellular localisation. Several theories exist, e.g. kin/recognition/oligomerization model of GA retention (Nilsson and Warren 1994) or the bilayer thickness model of Bretscher and Munro (1993) to explain the possible mechanism of GA retention. In both models, retention depends on a specific microenvironment. In the kin recognition/oligomerization model, it is hypothesised that both hetero- and homo-oligomers of GA proteins exist, and the inability of such complexes to enter the transport vesicles leads to retention in specific GA regions. Evidence for this mechanism has been

obtained by the isolation of GlcNAcT I/mannosidaseII hetero-oligomers (Nilsson and Warren 1994), galactosyltransferase homo-oligomers and sialyltransferase homodimers (Ma and Colley 1996).

In the bilayer model the transmembrane domain of the GA enzymes is critical for their retention in the GA, as this domain is shorter than the thickness of the cholesterol-rich transport vesicle membranes (Bretscher and Munro 1993).

However at the present neither of the models can accommodate all requirements for retention in the GA, and it is possible, that distinct, additive mechanisms account for the GA localisation of the resident enzymes.

3.5 Regulation of glycosylation

Several levels of regulatory mechanisms exist to modulate glycosyltransferase activities. Examples of transcriptional, translational, postranslational, hormonal and even calcium-dependent control mechanisms have been reported. Although these mechanisms are not sufficiently understood, a very complex, elaborate and often tissue specific regulatory network is emerging. Some illustrative examples are described next.

Transcriptional regulation

GlcNAcT V initiates the synthesis of tetra-antennary N-linked glycans (see Figure 3.4.,viii, p.35). The upregulation of these structures is commonly associated with malignant transformation.

As transformation typically alters the cellular proliferation-signalling pathway, it was reasoned, that some glycosyltransferases may be under the control of transcriptional activators which are part of the proliferation-signalling pathway. Indeed, Pierce et al. (1997) have shown that GlcNAcT V is a target of the Ets family of transcriptional activators. These factors stimulate cell proliferation and are known to be activated by the *src* signalling pathway.

Postranscriptional regulation

The enzyme initiating the synthesis of the dolichol-linked oligosaccharide precursor (see Figure 3.2, p.31) plays a crucial role in the overall control of N-linked glycosylation. The half-life of the yeast mRNA encoding this enzyme (ALG7) is regulated by growth stimulating agents, and according to the mitotic (cell cycle) stage of the yeast. It is not yet clear, whether similar mechanisms apply to higher organisms (Lennon et al. 1997).

Postranslational regulation

One of the most thoroughly studied glycosyltransferases is β 1-4galactosyltransferase. It is regulated in a tissue-specific manner, and two different transcripts are produced. One is constitutively expressed and is driven by the Sp1 promoter. In addition to the tissue specific expression and splicing the activity of this enzyme is regulated by phosphorylation. Removing the phosphate residues reduces enzyme activity approximately six-fold (Bunnell et al. 1990).

N-Acetyl-galactosaminyltransferase I, the first enzyme initiating O-linked glycan synthesis, has three potential N-linked glycosylation sites. Deglycosylation of the enzyme does not affect its transferase activity. However, if the three glycosylation sites are removed by targeted mutagenesis, no O-glycosylation can be detected in the mutant cell line. At least one of the potential N-linked glycosylation sites has to be occupied for O-glycosylation to occur. An explanation of these results is that the N-linked glycosylation is necessary for the GalNAcT to exit the ER, otherwise it is retained and eventually degraded (see also quality control, p.32 of the ER by calnexin/calreticulin and Piller [1997]).

Hormonal regulation

Oligosaccharyltransferase (OST) which transfers the oligosaccharide precursor to the protein and thus initiates N-linked glycosylation can be under hormonal control at least in

cultured porcine thyroid cells. Thyrotropin increases OST activity over 4 fold in this cell line (Desruisseau et al. 1996).

In the nervous system, corticosteroids can modulate the overall sialyltransferase activity. This action seems to be region specific, perhaps reflecting the expression of different sialyltransferases (ST) isoforms (corticosteroid responsive and nonresponsive). The hippocampal enzyme seems to be resistant to the action of glucocorticoids. A general trend from these studies was the reciprocal regulation of $\alpha 2$ -3 vs. $\alpha 2$ -6 ST activities. Where glucocorticoids enhanced $\alpha 2$ -3ST activity a corresponding decrease in $\alpha 2$ -6 activity was often noted (Coughlan et al. 1996).

Calcium and the synthesis of polysialic acid

In addition to the $\alpha 2$ -3 and $\alpha 2$ -6 linked sialic acids, the CNS is well known for its expression of a unique $\alpha 2$ -8 linked sialic acid polymer, polysialic acid. This molecule negatively modulates the homophilic adhesion properties of N-CAM, and also affects the cell surface properties of other cell adhesion molecules (Kiss and Rougon 1997). In a study by Bruses and Rutishauser (1998) the N-CAM polysialylation state was found to be dependent on the intracellular calcium pool. Moreover the PST activity was sensitive to changes in calcium concentration *in vitro*.

These are only patchy data, reflecting our current understanding. No conclusions can be drawn on the basis of these individual examples, however it is likely that glycosyltransferase regulation can respond to the special needs of individual tissue types it is expressed in. For example, hormones may regulate glycosylation in a hormone secreting cell line, calcium may modulate PSA synthesis in the nervous system in addition to the more general regulatory mechanisms.

3.6 *The glycosylation is over, what next?*

What happens to the glycoproteins when the final glycosylation step is over? How do they leave the GA and most importantly how do they reach their correct destination? These are especially interesting questions in polarised cells, like neurons. Some insights into these mechanisms were gained by site directed mutational studies. The rationale behind these studies came from the similarities between the epithelial and neuronal protein sorting mechanisms. Some proteins were directed preferentially to the apical surface, some to the basolateral membranes of epithelial cells. The same proteins when transfected to neuronal cells were localised to axons or dendrites respectively. This would suggest that similar sorting/transport mechanisms might operate in all polarised cells. Two mechanisms have been suggested to account for the **apical** sorting pathway:

- sphingolipid-cholesterol rafts forming within the exoplasmic leaflet of the Golgi membranes (Keller and Simons 1997)

mannose-rich N-linked glycans (Schelffele et al. 1995)

Basolateral sorting was correlated with:

- tyrosine residues in the context of at least one hydrophobic residue
- a motif grouped around a leucine/leucine or leucine/isoleucine pair
- an as yet undescribed mechanism, different from the first two

These signals (motifs) of the “passing through” proteins are most probably recognised by specific adapter proteins, which are associated with clathrin coated vesicles, or with other, non-clathrin transport vesicles. These proteins are recycled between the Golgi reticulum and the destination membrane through an unknown mechanism. The specific composition of the adapter protein complexes determines the type of glycoprotein cargo and possibly some aspects of the destination as well. AP-1, AP-2, AP-3, auxilin and p140 are examples of the recently identified adapter proteins. The latter three are specifically expressed only in the brain, reflecting perhaps the special requirements of this tissue. AP-1 is associated

with glycoprotein transport from the trans-Golgi compartment, AP-2 with the transport and recycling of plasma membrane proteins (like endocytosis of receptor-ligand complexes, or recycling of membrane proteins), and AP-3 have been found in the brain associated with transport vesicles. AP-3 may receive further attention on the basis of its decreased expression in the brains of Alzheimer patients, more specifically its O-GlcNAcylated isoform (Yao and Coleman 1998).

Endocytosis and recycling may not only be important for ligand-transporting receptors, like neurotrophins and their receptors, but also for CAMs, like integrins which are involved in cell motility. CAMs, which have been translocated to the rear end of the leading edge (of the growth cone for example) by the backward actin flow, are endocytosed and recycled to the leading edge. The internalization and recycling of CAMs generates a gradient of adhesive strength, which is thought to be necessary for cell locomotion (Lauffenburger and Horwitz 1996).

L1 is unique among the cell adhesion molecules, that its adhesive properties are not modulated by cytoplasmic phosphorylation (unlike integrins or cadherins). Very recently it has been demonstrated that the adhesive properties of L1 in the growth cone are regulated by AP-2 mediated recycling in clathrin coated vesicles. The endocytosis occurs at the rear end of the growth cone (Kamiguchi and Lemmon 1998). As the composition, concentration and distribution of CAMs is integral to the mechanisms of neurite motility and synaptogenesis, regulated endocytosis may be a mechanisms so far overlooked in regulating these processes.

In summary to this section, it can be concluded that protein sorting mechanisms exist in every eukaryotic cell. The transport of glycoproteins occur via specific transport vesicles (clathrin-based, non-clathrin based; and COPI-vesicles in the ER and GA). The cargo of these vesicles is determined by the specificity of the adapter proteins. *N-linked*

glycosylation is one of the targeting mechanisms. Much remains to be understood about the actual transport of these vesicles to the proper subcellular compartments.

4. General features of central nervous system glycosylation

One of the most characteristic features of the brain is its seemingly incomprehensible complex wiring. This is in large part due to the unique properties of its two main cell types, the glia and neurons.

At the molecular level as well, many proteins (and/or isoforms) are only expressed in the brain. This is achieved through neural restrictive silencer elements in the genes of these proteins and the proteins recognising these sequences, the neural restrictive silencer factor (NTSF) or the RE-1-silencing transcription factor (REST) (Edelman and Jones 1998).

Even the large number of specialised cells and molecules present only in the brain, could not by themselves account for the complexity and selectivity of synaptic connections, the development of specialised brain regions etc. Glycosylation may provide, and indeed does provide another level of specific information when presented on proteins, lipids or proteoglycans. However, as is the case with the specialised cell types, and a large number of brain-specific molecules, is there evidence for “brain-specific” glycosylation? And if yes, what might be their functional importance/relevance? These questions will be discussed in some detail next.

Since the first glycoanalytical studies by Krusius (1977, 1978a, 1978b), it was suspected that different tissue types of the same animal may have distinct oligosaccharide profiles. This included the brain as well, and further characterisation of its glycoconjugates has lead to several important observations.

First of all it was the description of α 2-8 linked sialic acids by Finne and Krusius in 1977 (Finne et al. 1977), which was later identified as polysialic acid present mostly on the neural cell adhesion molecules N-CAM (Finne 1982a). Next, the analysis of terminal sugar residues indicated an unusually high concentration of fucosylated glycans (Krusius 1977). This fucose was later localised to peripheral structures in α 1-3 linkage to Gal β 1-4GlcNAc,

which is the X or Lewis X determinant present predominantly in its sialylated form (sLeX) (Krusius 1978a). Also a significant proportion of N-linked oligosaccharides had α 1-6 linked fucose to the proximal glucosamine at the reducing termini (core fucose). The most abundant neutral N-linked structures turned out to be the oligomannosidic glycans, which were first suspected by the large proportion of concanavalin A binding glycoproteins in the brain (Gurd 1989) and recently confirmed by Chen et al. (1998). Man 5-9 structures are all present Man5 being the most abundant throughout development.

Many neural cell adhesion molecules as well as glycolipids and proteoglycans were found to carry the negatively charged HNK-1 sulphoglucuronyl epitope ($\text{SO}_4\text{-3GlcA}\beta\text{1-3Gal}\beta\text{1-4GlcNAc}$). Another characteristic of the anionic oligosaccharides according to Finne (1990) is the prevalence of α 2-3 linked as opposed to α 2-6 linked sialic acid at the terminal positions of multiantennary N-linked oligosaccharides.

In retrospect it is fascinating, that the four well-known and widely used monoclonal antibodies (L2, L3, L4 and L5) raised against the mouse brain membrane glycoproteins and the L1 antigen by M. Schachner's group turned out to recognise many of these "specific features" of the nervous system glycosylation. The L2 antibody (also called L2/HNK1), as its name implies, reacts with the HNK-1 epitope. L3 and L4 antibodies bind to neuronal cell surfaces inhibit cell adhesion and neurite outgrowth and interfere with the interaction between L1 and N-CAM. The antibodies recognise oligomannosidic structures (Schmitz 1993). And finally, the L5 epitope almost certainly reacts with the LewisX trisaccharide determinant (Streit et al. 1996).

Therefore at present, the most characteristic features of the brain N-linked glycosylation can be summarised as:

polysialic acid
α 2-3 linked terminal sialic acid
HNK1-epitope
oligomannosidic glycans
core fucose
bisecting GlcNAc
Lewis X epitope

This is an arbitrary and subjective listing of certain protein-linked glycan structures, and in itself, outside (neuro)biological context is not useful. However a closer examination of the biological phenomena these sugars are involved in may give us some clues about their versatile role in the nervous system.

Polysialic acid (PSA)

Since its first description by Finne (1982a), polysialic acid has become perhaps one of the best known glycan epitopes in the scientific community. Its unusual structure (high negative charge), the availability of monoclonal antibodies and a highly specific endosialidase (endoN) made the understanding and characterisation of its biological functions possible. PSA is found almost exclusively on the cell adhesion molecule N-CAM although there has been one report about its presence on the sodium channel alpha subunit (Zuber et al. 1992). The expression of PSA is developmentally regulated, closely correlated with aspects of axonal pathfinding, targeting and muscle innervation (Rutishauser and Landmesser 1996, Seki and Rutishauser 1998, Storms et al. 1996). The biophysical properties of PSA would suggest its mode of action, i.e. a bulky, negatively charged residue with considerable hydration volume would in some respects limit the intercellular interactions in its immediate environment or even the *cis*-interactions of N-CAM within the membrane. Although the *exact* mechanisms of its action are not known, it is suggested, that PSA may be involved in nerve branching, fasciculation, and activity dependent

synaptic plasticity. Polysialic acid is downregulated in the adult, with the exception of hippocampus, where it is expressed throughout adulthood (Seki and Arai 1993, Seki and Arai 1991).

Removal of the polysialic acid with endoN prevented the induction of LTP and LTD in the CA1 region of hippocampal slice cultures (Cremer et al. 1994, Muller et al. 1996), without affecting other cellular or synaptic parameters such as resting or action potential. Also, intrahippocampal injection of endoN impaired spatial learning of rats in the Morris water maze (Becker et al. 1996). An increase in N-CAM polysialylation was reported by Doyle et al. (1992a) in rats after a passive avoidance training task. These all confirm the involvement of polysialic acid perhaps in conjunction with N-CAM in certain aspects of synaptic plasticity relevant to learning and memory (see also p.24).

The biosynthesis and regulation of PSA activity have also been intensively studied. Two different polysialyltransferase enzymes, ST8SiaII/STX and ST8SiaIV/PST from four different species (Kojima et al. 1995, Eckhardt et al. 1995) have been cloned to date.

The regulation of polysialic acid synthesis has been mentioned on p. 46, (Regulation of glycosylation) briefly, it can respond to changes in calcium concentrations, and in this way to be regulated by synaptic activity (Bruses and Rutishauser 1998). Modulation of the enzyme activity at mRNA level has also been suggested (Eckhardt et al. 1995, Muhlenhoff et al. 1996).

Other polymers of sialic acid, the 2-keto-3deoxy-D-glycero-D-galacto-nononic acid, poly-KDN, poly-deaminoneuraminic acid residues (Qu et al. 1996; Ziak et al. 1996) are present in a regulated fashion in the kidney and lung during development. This may suggest that other polymers of negatively charged monosaccharide residues may have similar functions in a wider concept of organogenesis.

α 2-3 vs. α 2-6 linked sialic acid

Sialic acids are always in terminal position, and hence are the first to be encountered by extracellular receptors. A large number of endogenous molecules with lectin-like properties and affinity for terminal sialic acids have been described recently. They are the selectins, sialoadhesins and other as yet unclassified molecules (Schauer et al. 1995). Some of these molecules belong to the immunoglobulin superfamily and in 1994 they have been dubbed the sialoadhesin subfamily of the Ig superfamily by Crocker, Schnaar, Schauer and co-workers (Kelm et al. 1994). Myelin associated glycoprotein, MAG also belongs to the family of sialoadhesins and it recognises terminal sialic acid α 2-3 linked to subterminal Gal β 1-3GalNAc on gangliosides, with the highest affinity for GQ1b α . Other members of the sialoadhesin family, e.g. CD22 specifically binds to NeuAc α 2-6Gal β 1-4GlcNAc, whereas sialoadhesin recognises NeuAc α 2-3Gal β 1-3(4)GlcNAc or NeuAc α 2-3Gal β 1-3GalNAc. Therefore a suggestion from these studies is that the fine molecular details of terminal sialylated structures can be "read" by specific lectin(-like) molecules (Kelm et al. 1996; Schnaar et al. 1998; Varki 1997).

In the nervous system only two sialoadhesins have been described so far, the MAG and the Schwann cell myelin protein (SMP). It is not yet known, whether the sialic acids present on other glycoproteins could also serve as ligands for MAG and SMP or similar receptors, but on the basis of the above mentioned observations it is expected to be the case.

HNK-1 (Leu-7) epitope

The HNK-1 epitope (p.51) is widely expressed on a large number of neural cell adhesion molecules, including N-CAM, L1, MAG (myelin associated glycoprotein), Ng-CAM (neuron-glia cell adhesion molecule), Nr-CAM (Ng-CAM related CAM), TAG-1 (transient axonal glycoprotein 1), F11/F3, P0, SAG (a 170 kDa glycoprotein associated with PNS myelin and Schwann cells), MOG (myelin/oligodendrocyte glycoprotein), PMP-22

(peripheral myelin protein 22) and extracellular proteins like astrochondrin, J1, integrins and ependymins (Olsen et al. 1993, Nair et al. 1998). They are all involved in specific cell-cell interactions, indicating a special role for the HNK-1 epitope. HNK-1 may be involved in the homophilic binding of P0, a glycoprotein responsible for the formation and maintenance of the myelin sheet. The homophilic adhesion is thought to be mediated by a lectin like interaction between P0 and the HNK-1 (Griffith et al. 1992).

The exact structure of the epitope has been recently determined (Voshol et al. 1996) and the enzymes involved in its biosynthesis (glucuronyltransferase and sulphotransferase) cloned (Terayama et al. 1997, Chou and Jungalwala 1993, Chou and Jungalwala 1996).

In addition to mediating homophilic adhesion between P0 in the myelin sheath, it was suggested that the HNK-1 epitope might act as a ligand for other cell adhesion molecules (Kunemund et al. 1988). In their experiments the HNK-1 tetrasaccharide -without being attached to glycoprotein or glycolipid- inhibited cell-cell and cell-substrate interactions. More recently, a single 30 kDa protein was affinity purified on sulphoglucuronylglycolipid (SGGL) columns. The protein, named SBP-1 (SGGL binding protein 1) is homologous to amphoterin, a neuron outgrowth promoting protein (Rauvala and Pihlaskari 1987; Wartiovaara et al. 1990). The expression pattern of SBP-1 is very similar to SGGLs, suggesting that interactions between sulphated sugar epitopes and their endogenous receptors might occur during prenatal brain development (Nair et al. 1998).

Oligomannosidic glycans

Another group of oligosaccharides, the neutral, N-linked oligomannosidic glycans seem to be a characteristic feature of the neuronal cell membranes, including synaptic junctions. Relatively little is known about the distribution and function of these glycans compared to the previously mentioned glycan epitopes.

That they are quantitatively the dominant components of the brain glycans has only been confirmed recently (Chen et al. 1998), where they accounted for approximately 15% of the

total N-linked glycan pool. Also in the chicken pure synaptosomal membrane fraction they are the most abundant structures (see Neutral glycan profile, p.113). An important insight about the potential function of this “least processed” (in terms of biosynthesis, Figure 3.4, p.35) class of N-linked oligosaccharides was gained by the use of already mentioned L3 and L4 monoclonal antibodies. A number of cell adhesion molecules carry the L3 and L4 epitope: L1, MAG, P0, Thy-1, AMOG and several unidentified glycoproteins (Schmitz et al. 1993, Fahrig et al. 1990). In adhesion assays, the L3 antibody has been shown to inhibit neuron-neuron adhesion only (Kucherer et al. 1987), whereas the L4 antibodies inhibited neuron-astrocyte adhesion as well (Fahrig et al. 1990). Also, L3 interfered with the heterophilic interactions between N-CAM and L1, indicating that oligomannosidic glycans present on L1 (not on N-CAM) may act as ligands for N-CAM. Indeed, a C-type lectin carbohydrate recognition domain (CRD) has been identified within the 4th immunoglobulin domain of N-CAM, homologous to a human mannose binding lectin (Horstkorte 1993). Moreover, oligomannosidic glycans when added to the culture medium of cerebellar neurons drastically inhibited neurite outgrowth.

A similar function for specific carbohydrates, including oligomannosidic structures during nervous system development (in leech) has also been proposed by Zipser and Cole (1991) and Song (1995). The full set of leech sensory afferent neurons can be identified via the expression of a surface carbohydrate marker, called C0, which is an oligomannosidic glycan (Zipser and Cole 1991). Antibodies against C0, mannose-BSA neoglycoproteins and N-glycanase treatment have all inhibited the defasciculation of sensory afferents in the CNS neuropil. The C0 was present on a single, 130 kDa glycoprotein, the identity of which is still unknown. It is possible, that the C0 epitope is recognised by mannose-specific endogenous lectin like receptors.

Although its relevance to the C0 epitope is unknown, an endogenous, mannose binding lectin has been isolated from rat cerebellum by Zanetta et al. (1985). It has been postulated

by the authors (Dontenwill 1985) that the lectin is involved in recognition mechanisms followed by internalization into the Purkinje cells of parallel fibre glycoproteins as a first step of synaptogenesis. In later experiments using a different paradigm, that of the neuromuscular junction formation, a similar process was observed (Thomas et al. 1994).

In summary, it is expected that a whole range of mannose specific receptors may exist in the nervous system, and in conjunction with the oligomannosidic glycans present on a large number glycoproteins mediate many of the developmental processes.

Bisecting GlcNAc and core fucose

Bisecting glucosamine residue and α 1-6 linked core fucose are two other characteristics of the “brain-type” glycosylation. They are discussed together, first because there is little known about the biological effects of these structures in general, and secondly, as they are not in terminal positions on glycan structures, these sugar residues are possibly not directly recognised by other molecules as the above mentioned carbohydrate epitopes.

Very few studies have directly addressed the role of bisecting GlcNAc. It is known that the introduction of GlcNAc in β 1-4 linkage to the β -mannoside of the trimannosyl core affects the conformation of sugar chains. Once the bisecting GlcNAc has been introduced, other glycosyltransferases such as GlcNAcT-II, GlcNAcT-IV, GlcNAcT-V and β 1-4GalT are no longer able to act on the biantennary sugar chains. In this respect, GlcNAcT-III and the bisecting GlcNAc are key modulators of glycosylation (Taniguchi and Ihara 1995).

Indirect evidence strongly suggests a biological function for this product of glucosamintransferase III. Sultan et al. (1997) noticed a differential sorting of certain glycoproteins in a hepatoma cell line upon the upregulation of GlcNAcT III activity and hence the bisecting GlcNAc levels. Alterations in the ratio of bisecting structures have also been noted after transformation especially in the liver, where there is virtually no GlcNAcT-III mRNA present under normal conditions.

On the basis of similar considerations a mouse strain deficient in GlcNAcT III was created by Priatel et al. (1997). The enzyme is encoded by a single allele gene and is highly conserved throughout evolution (more than 90% homology between the mouse and human copy). Unexpectedly, no severe or even mild phenotypic consequences were evident up to 12 month of age. The mice did not express bisecting GlcNAc as detected by lectin staining, and the morphology and physiological parameters of the kidney (where bisecting GlcNAc is abundant) were normal. Therefore it appears that GlcNAc T III is dispensable for the normal development, viability and reproduction of the mice.

The effect of *core fucose* on the glycan conformation has been studied by a fluorescent energy transfer method. The application of this unique technique has clearly demonstrated that the presence of core fucose had a dramatic stabilising effect on the extended conformation of the Man α 1-6 arm of a biantennary oligosaccharide (Stubbs et al. 1996). Only one single glycan structure has been studied, but on the basis of their results, it can be suggested that core fucosylation in general, can modify the conformation of peripheral structures. This, in turn may influence the interaction of these glycans with carbohydrate receptors or even the accessibility of the proteins to proteolytic enzymes.

LewisX epitope

Surprisingly little functional information is available about this carbohydrate epitope in the brain despite its high abundance there. CD15 (also known as X, LewisX, FAL or SSEA-1) was initially detected on embryonal carcinoma cells and in preimplantation mouse embryos from the 8-cell to the 32-cell stage. Later several monoclonal antibodies were generated, which all recognized the same trisaccharide, 3-fucosyl-N-acetyllactosamine, and were assigned the "Cluster of Differentiation 15" by the International Leukocyte Typing Workshops (Gooi et al. 1981). CD15 reactive epitopes are widely expressed in non-neural tissue types, including the epithelial cells of the digestive and urinary tract, the reproductive system, some endocrine glands, skin appendages and myeloid cells. The

trisaccharide was also found in malignant tumors and during specific stages of embryogenesis, therefore CD15 is regarded as an onco-developmental antigen.

A closer investigation of the LewisX epitope during embryogenesis and the difficulty in finding a receptor for this molecule lead Hakomori and his colleagues to the suggestion, that perhaps LewisX is the receptor itself, i.e. LewisX can mediate homophilic carbohydrate interactions. This has been further supported by several independent studies showing that LewisX positive liposomes or plastic beads can aggregate or segregate out from a mixture of liposomes (Eggens et al. 1989, Kojima et al. 1994). Also, cell lines modified to display LewisX on their surface acquire the ability to form stable cell-cell contacts (Boubelik et al. 1998). Lewis X has also been crystallised, and the possible interaction between two opposing LewisX molecules confirmed this interaction was the thermodynamically most favourable arrangement, as maximal number of hydrogen bonds was formed (Perez et al. 1996). Molecular modelling of LewisX has shown that one surface of the molecule is more hydrophobic than the opposite surface, and that the interaction between two molecules preferably involves the interaction between the two hydrophobic surfaces (Kojima et al. 1994). Bivalent cations, like calcium or manganese are absolutely required for this interaction, and mangan seems to further enhance the binding. In this respect it is interesting that calcium chelates with two molecules of L-fucose (Cook and Bugg 1975).

Distribution of CD15 in the CNS

Rather little is known about the function of CD15 in the nervous system, despite the extensive immunohistochemical data. On the basis of those data, no simple function is evident for CD15 positive glycoconjugates, though they might be involved in the patterning of specific brain regions. In the adult nervous system of various species CD15 is present mostly on glial and certain neuronal cells. During development of the CNS, the expression of CD15 is regulated in a temporal and spatial manner on both glial and

neuronal subpopulations (Sato and Kim 1994, Mai et al. 1998, Ashwell and Mai 1997a; Ashwell and Mai 1997b; Ashwell and Mai 1997c; Ashwell and Mai 1997d). In the hippocampus, CD15-positive glial cells are concentrated within the molecular layer of dentate gyrus and in the layer of the pyramidal cells, though other brain regions are stained as well (Gocht et al. 1996).

Amongst the antibodies produced against the glycoproteins of the murine nervous system (L2, L3, L4 and L5), L5 recognises LewisX (Streit et al. 1996). The exact structure of the antigen is not yet known. Several cell adhesion molecules (L1, Thy-1) and the chondroitin sulphate proteoglycan astrochondrin are L5 positives. The L5 antibody interferes with neural induction in vivo (Roberts et al. 1991), and it has been suggested that the L5 epitope or the L5 positive molecules might be directly involved in neural induction.

The L5 epitope however shows some differences in its staining pattern when compared to other monoclonal anti-CD15 antibodies. A large number of these antibodies is available now, and in a study addressing the relative specificity of these antibodies, it was concluded that they show different binding affinities in the same assay system (flow cytometric analysis, FACS, Vlasova et al. 1996). This observation makes the interpretation and comparison of different studies difficult. It is evident from a recent study by Chen et al. (1998), that Lewis X epitope can be present on a very large number of different hybrid or complex glycans. The antibodies probably do not strictly differentiate between the different presentations of LewisX. Further experiments are therefore required to specify in more detail the exact structure of the carbohydrate antigens recognized by the individual monoclonal antibodies.

In conclusion, the high concentration of LewisX present in the brains and the intrinsic property of these carbohydrate structures to aggregate in the presence of calcium and/or magnesium may suggest an adhesive role in the nervous system. Such a carbohydrate-carbohydrate interaction possibly does not mediate strong binding. In certain well-

understood biological processes, like embryogenesis and inflammation, the initial carbohydrate-carbohydrate interactions, if successful, are further strengthened by other protein mediated adhesive interactions. For example, during the aggregation of morula-stage embryos the initial LewisX-LewisX interaction can lead to further strengthening through cadherin mediated adhesion. Similarly, during leukocyte trafficking through the endothelial cells the initial E-selectin mediated recognition of the sLeX epitope on the leukocyte surface is reinforced by an integrin dependent stronger adhesion.

Carbohydrate mediated interactions are generally weaker than protein-protein interactions, but biologically significant adhesion can be achieved through the multiple presentation of sugar epitopes, as has been demonstrated for E-selectin inhibitors (Stahn et al. 1998). LewisX is present on proteoglycans and polylactosamine chains, the long polymeric chains of these sugar backbones would be ideally suited for multivalent LewisX presentation.

5. Behavioural and biochemical methods

5.1 Training on a passive avoidance task

Ross Chunky chicks of both sexes were purchased as eggs and hatched in our laboratory. They were held in communal incubators on a 12-h light/dark cycle (38-40°C) until 24 hours post-hatch. On the morning of each training day, chicks were taken to the training room and housed in pairs in a metal box illuminated by a 25W red bulb. After a period of equilibration (40-60 minutes) to the training room, chicks were pretrained by three, 10 second presentations of a small (2.5 mm) white bead. The bird's behaviour was scored as "peck" or "nonpeck". Birds that pecked during pretraining were trained by a single 10 second presentation of a larger chrome-bead (4 mm) dipped in a bitter tasting methylanthranilate (MeA) (Figure 5.1). The bird's behaviour was scored again as peck or nonpeck. Nonpecking birds were not used for further analysis (5-15% of chicks).

Nine hours after training chicks were tested with a dry chrome bead. Birds avoiding the dry bead (representing learning and retention of the task) were used for further analysis, and those pecking at the dry bead were not further analysed.

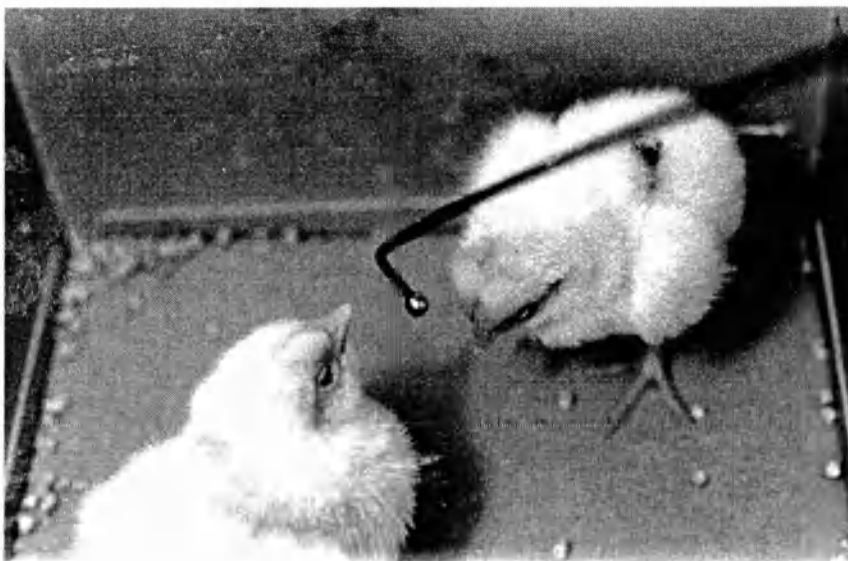


Figure 5.1. Passive avoidance training in day-old chickens

Same hatch quiet chickens housed in the same training room under identical conditions (i.e. in pairs) but without any behavioural training were used as “quiet controls”, and both groups (MeA and Quiet) were processed in parallel. No water trained animals were used in this study.

As will be described later, two different membrane preparations were necessary during the course of these studies. In the first, forebrains were used for pure synaptosomal membrane prep, in the second a small brain region implicated in memory formation for the passive avoidance training task, the intermedial medial hyperstriatum ventrale (IMHV), was dissected and the crude synaptosomal membranes (the P2 fraction) analysed.

5.2 *Pure synaptosomal membrane preparation from whole brains*

Pure synaptosomal membrane fractions were prepared by the method of Jones and Matus (1974). Despite its name the method does not result in literally “pure” synaptosomal membrane preparations, the term has been coined to indicate that this method provides one of the “purest” preparations. The purity of this prep typically does not exceed 75-80% (Margolis and Margolis 1977), as evidenced by electron microscopic and biochemical studies of the different fractions obtained after the density centrifugation step. The most common sources of contamination are the mitochondria, myelin sheath fragments, glial and microsomal membranes. The SPMs prepared during the course of these studies were checked by electron microscopy (Figure 5.2), and were found to be at least 85 % pure (Heather Davies, personal comm.). This relatively high purity could be due to the fact, that myelination is not yet completed in day-old chickens and hence contamination from myelin is relatively low.

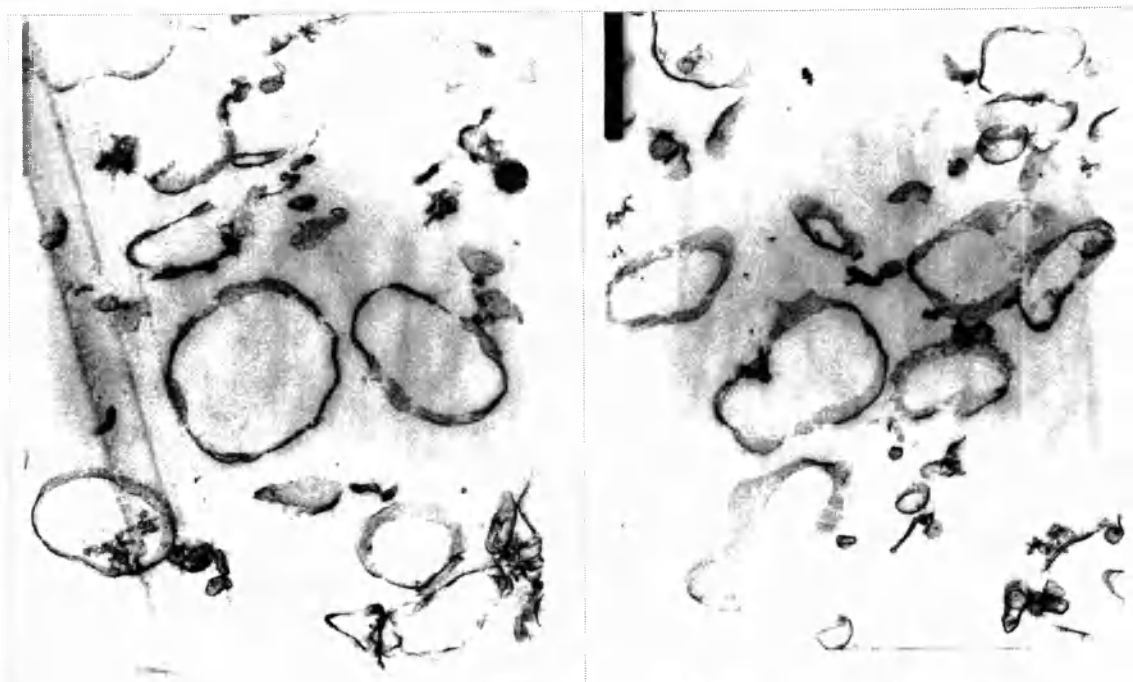


Figure 5.2. Electron micrograph of the pure SPM preparation used in this study, magnification: x20,000. (courtesy of Heather Davies)

Animals were decapitated 9 hours after training and the brains (forebrain without the midbrain and cerebellum) dissected. The reasons behind choosing the 9 hours time-point for sample preparations were in the observations of the two waves of protein and glycoprotein synthesis (Freeman et al. 1995). During the second wave (4-6 hours after training), proteins are synthesised and then glycosylated (5.5-8 hours) as part of the memory consolidating process. The second wave is associated with long-term memories, as it does not occur after “weak-training” (Rose 1996). Therefore it was assumed that the actual glycosylation step, when the fucose is being incorporated into the glycan structures, takes place during this time window. However, these glycoproteins have to be delivered to the synaptic plasma membranes, because they are presumably synthesised in the Golgi apparatus. Although the time required for this delivery is unknown, a 3 hours time window for this transport to occur has been allowed, therefore the 9 hours time point (6 + 3) for sample preparation.

Certain modifications to the original method published by Jones and Matus (1974) were necessary due to differences between the properties of day-old chicken and adult rat brain

synaptosomal plasma membranes. These included increasing the density of sucrose gradient and the high-speed centrifugation time.

Forebrains were homogenised in 0.32M sucrose/10 mM HEPES pH 7.4 in the presence of proteinase inhibitors (Boehringer) using glass homogeniser (0.25 mm clearance). The homogenates were centrifuged at 3000 rpm (1000 g, 4°C) for 15 minutes in a Beckman JA-1 rotor. The supernatant was pelleted again at 11.500 rpm for 15 minutes. The resulting pellet (P2) was lysed in minimal volume of lysis buffer (5 mM HEPES pH 7.4) for 1 hour on ice. The lysate was then homogenised in a Potter homogeniser and diluted to a solution of 38% sucrose using 48% sucrose solution (buffered with HEPES to pH 7.4). Onto the top of this solution, an equal volume of 28.5% sucrose solution (pH 7.4) was carefully layered, which was then overlaid with a thin layer of 10% sucrose. This gradient was ultracentrifuged at 21.5 krpm in an SW28 rotor for 3 hours at 4 °C. The interface between the 28.5 and 38% sucrose was carefully removed and resuspended in maximal volume (depending on the centrifuge and the tubes used) of 5 mM HEPES pH 7.4 containing 5 mM CaCl₂. This solution was pelleted at 80 000 g (34 000 rpm T7.I.Ti Beckman rotor) for 30 minutes, and the procedure repeated again. The final pellet was used for the extraction of glycoproteins.

On a single training day typically 36 chickens were used for the membrane prep. Several preparations were pooled in order to increase the level of membrane material for glycoprotein extraction. The average yield of this procedure was 35mg membranous material (“pure” SPM) per 10 grams wet brain tissue, approximately 0.3-0.4% yield.

5.3 Glycoprotein extraction with Triton-X

Glycoproteins were prepared from the pure synaptosomal membrane fraction by detergent (Triton-X) extraction. For optimising recovery, five different detergent concentration (0.1%, 0.5%, 1%, 2% and 3%) were tested. Increasing the detergent concentration above

2% did not increase the protein yield any further; therefore the 2% final concentration was used during the extraction procedure.

Pure synaptosomal plasma membrane pellets were resuspended in extraction buffer (final concentration 5-8 mg/ml). Appropriate volume of 5 x concentrated solubilization buffer (250 mM HEPES, pH 7.4, 0.75 M KCl) containing 10% Triton-X was then added to give 2% final detergent concentration. This mixture was rotated by a rotating mixer at 4 °C for 1 hour and pelleted at 38 000 rpm/100 000g (Ti.I.70 rotor) for 1 hr. The supernatant containing detergent solubilized material was precipitated with 3 volumes of ice-cold ethanol at -40 °C for several hours and recovered by centrifugation at 15 000 rpm (JA-1). The pellet was resuspended and centrifuged repeatedly (3 times) to remove Triton-X. The final pellet was cryogenically dried for 12 hours and used for glycan release in GlycoPrep 1000.

Protein assay of the ethanol precipitated material (using the BioRad protein assay kit following the manufacturer's instructions) revealed ~60% w/w protein content. The contamination was probably due to (polar) glycolipids, like gangliosides, although this did not affect the results because the carbohydrate residues released from these glycoconjugates were destroyed during the hydrazinolysis step. Therefore in the second set of experiments a method previously developed by Finne (1982b) for rat brain glycoprotein isolation was applied, where the membrane fraction was delipidated using organic solvents.

5.4 IMHV dissection and crude synaptosomal membrane preparation

IMHVs were manually dissected 9 hours after training. The region is illustrated on Figure 5.3. Following decapitation the skin was pulled over from the neck towards the eyes, thus exposing the skull. As the skull is relatively soft at this age, small, sharp surgical scissors were used to remove the top of the skull by cutting carefully around the brain from the posterior towards the anterior regions from both left and right sides. The exposed brain was

gently divided into the right and left hemispheres by medium sized spatula. Since the hyperstriatum ventrale is situated directly under the ventricles, the soft roof tissue was gently pulled over by a very small spatula exposing the hyperstriatum ventrale (steps ii-vi on Figure 5.3.b.). The IMHV was cut out using a small sterile surgical blade as illustrated on Figure 5.3.b step vii.

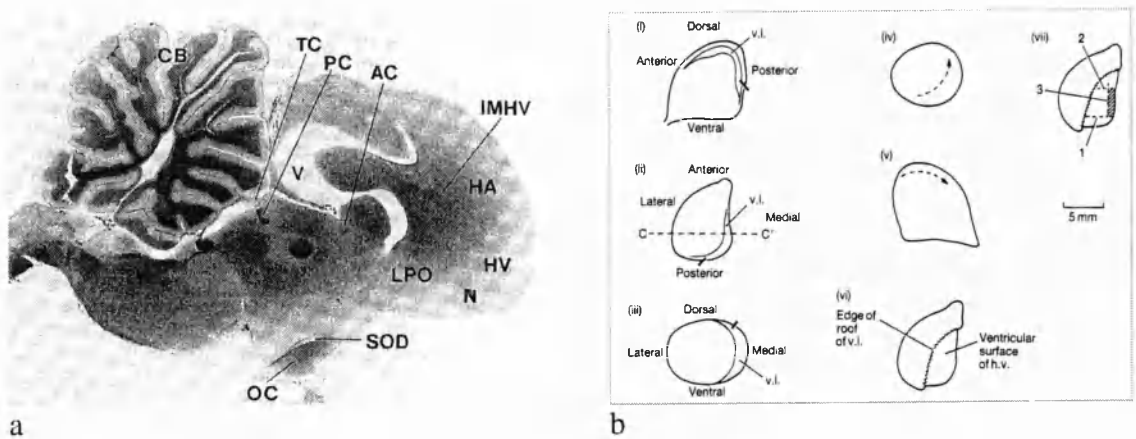


Figure 5.3. Sagittal section of a two-day old chicken brain. TC-tectal commissure, PC-posterior commissure, AC- anterior commissure, SOD-supra-optic decussation, OC-optic chiasma, CB-cerebellum, V-ventricle, IMHV-intermedial medial hyperstriatum ventrale, HA-hyperstriatum accessorium, HV-hyperstriatum ventrale, N-neostriatum, LPO-lobus parolfactorius (from Rogers 1995) b.) Dissection of the IMHV as described by Horn (1991). See text for details.

The dissected left and right IMHVs were separately pooled on ice in 0.32M sucrose solution (pH 7.4) with protease inhibitors and crude synaptosomal membranes (P2 fractions) prepared. After measuring the weight of the pooled IMHVs (typically from 15-20 animals), appropriate volume of homogenisation buffer was added to give 10% w/vol. final concentration. The tissue was then homogenised in a glass homogeniser by 7 strokes at 700 rpm, then paused on ice for 30 seconds and further homogenised by another 7 strokes. The homogenate was centrifuged at 3000 rpm (1000g) for 15 minutes in a Beckman (JA-1) rotor at 4 °C. The supernatant from this centrifugation (S1) was centrifuged at 11.500 rpm for 20 minutes. From the resulting pellet (P2 fraction) glycoproteins were prepared by the method of Finne (1982b).

5.5 Delipidation of the crude synaptosomal membrane fraction

To 3 volumes of the aqueous suspension of P2 (1-5 mg/ml) 8 volumes of methanol was added, followed by 4 volumes of chloroform at continuous stirring. The sample was gently stirred for 30 minutes and centrifuged at 15 000 g for 15 minutes. The pellet was resuspended in water-methanol-chloroform (3:8:4) and centrifuged again. The solvents were then removed from the pellet with absolute ethanol and the pellet used for glycan release after lyophilization.

5.6 Chemicals and materials

Iodoacetamide	Sigma
Dowex AG-3	BioRad
Dowex AG-50 X-8	BioRad
C ₁₈ resin was from	Fischer
Acrylamide	BioRad
Bis-acrylamide	BioRad
Tetramethylethylenediamine (TEMED)	Sigma
Dithiothreitol (DTT)	Sigma
Sucrose	BDH
Triton-X	Sigma
HEPES	Sigma
Methylantranilate	Sigma
Protease inhibitor cocktail	Boehringer
CaCl ₂	Sigma
KCl	Sigma
EDTA	Sigma
Dithiothreitol	Sigma
NaCl	Fisher
Tris-HCl	Sigma
Na ₂ HPO ₄	Fisher
MgCl ₂	BDH
Trifluoroacetic acid (anhydrous, protein sequencing grade)	Sigma
10 x Tris/Glycine/SDS gel buffer (0.25M Tris, 1.92M Glycine, 1% SDS)	National Diagnostics

NaOH for HPAEC	BDH
NaHCO ₃	Fisher
NaOAC.3H ₂ O	Fisons
Sodium dodecyl sulphate (SDS)	Pharmacia Biotech
NaN ₃	Sigma
Acetonitrile (HPLC grade)	Rathburn Chemicals
Ethanol (95%)	Romil
Ethanol (absolute, 98%)	Fisher
Methanol	Fisher
Methanol (dried distilled)	Fisher
Triethylamine	Aldrich Chemicals
Glacial acetic acid (~100%)	BDH
Ethanolamine	Sigma
Formic acid (98-100%)	Fisons
Hydrochloric acid (standard grade)	Fisher
Trifluoroacetic acid (redistilled, 99+%)	Aldrich
Chloroform	Fisher
KCl	Sigma

5.7 Accessories

Microcon microconcentrators (Amicon, Inc., Beverley, USA)
Mini-PROTEAN II cell (BioRad) electrophoresis apparatus

5.8 Glycoprep consumables

Reagent Kit (one bottle of each reagent for GlycoPrep 1000 is sufficient for 20 preparations).

N+O Glycan Columns

Reactor Vessels with screw caps

Collection Vials with screw caps

RAAM 2000 Neutral Sequencing Kit

RAAM 2000 Neutral enzyme array

RAAM 2000 Control N-Glycan

GlycoPure Water

RAAM Workstation

Glycan Sizing Column

Calibration Standard (7.5mg/vial of lyophilised dextran standard)

Kit for fluorescent oligosaccharide labelling with 2-aminobenzamide (2-AB) (Oxford GlycoSciences)

5.9 Others

Immobilon P – transfer membranes, 0.45 µm, PVDF, Millipore

Microfilters (for glycan clean up) 0.45 µm, CN, (Life Sciences International, UK, Ltd)

DAB – horse-radish peroxidase substrate (Sigma)

Complete TM Protease Inhibitor Cocktail Tablets (Boehringer)

5.10 Solutions

10 x PBS per 1L

NaH₂PO₄ 2.03 g

Na₂HPO₄ 11.49 g

NaCl 85 g

The pH of the 10x solution is 6.7-6.9. The pH of the 1x solution should be adjusted to 7.3-7.5.

10 x TBS per 1L

Tris 60.55 g

NaCl 85.2g

Electrophoretic transfer buffer per 1L (Western blotting)

Glycine 14.4 g

Tris-HCl 3.03

Methanol 150 ml (15% final conc.)

5.11 Polyacrylamide gel electrophoresis

Proteins isolated from the whole brains or the IMHV P2 fractions were separated by polyacrylamide gel electrophoresis (PAGE) according to (Laemmli 1970). Premade Nu-PAGE (Nowex) gradient gels (6-12%) were used. Samples mixed with appropriate volume of 2 times concentrated sample buffer (containing 4%SDS, 20% glycerol, 10% mercaptoethanol, 0.004% bromphenol blue, 0.125M Tris HCl pH 6.8) and heated for 10 minutes at 100 °C. Gels were run at 150 V constant voltage under reducing conditions in Tris/Glycine/SDS buffer (National Diagnostics).

5.12 Western blotting

Gels were electroblotted in a BioRad blotting chamber for 4 hours at 150 mV constant voltage. The transfer buffer contained 15% methanol, as this concentration was found to result in optimal protein transfer. PVDF Immobilon P (0.45 μ m, Millipore) membranes were activated in methanol prior to blotting. After transfer, the nonspecific binding sites were blocked by a solution of 5% BSA in TBS for several hours at room temperature or overnight at 4 °C. The primary monoclonal antibodies against CD15 (mouse IgG, Immunotech) and sialyl-LewisX (mouse IgM, Immunotech) were diluted to 5 μ g/ml in 0.5% BSA/TBS, and the blot incubated overnight at 4°C with gentle shaking. The solution was then discarded, the blot washed 3 times with 0.5% BSA/TBS for 10 minutes. The secondary antibody (conjugated with alkaline phosphatase) against mouse IgG or IgM (Sigma) was diluted 1:30 000 in 0.5% BSA/TBS and incubated for 2-3 hours at ambient temperature. The washing process was repeated again, however the second wash contained 0.025% Tween-20. Blots were visualised by the addition of insoluble alkaline phosphatase substrate BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate nitro blue, Sigma).

In experiments examining the nature of CD15 positive structures, samples were digested before Western blotting with sialidase, endo- β -galactosidase, keratanase II or chondroitinase ABC. The following incubation conditions were used:

endo- β -galactosidase (Oxford GlycoSciences): 50 mM sodium acetate pH 5.8 containing 1 mM NaCl, 125 mU/ml, 18 hours/ 37 °C

sialidase (*Arthrobacter.ureafaciens.*, Oxford GlycoSciences): 10mM ammonium acetate pH 6.5; 2U/ml; 18 hours/ 37 °C

keratanase II (Seikagaku): 100 mM NaOAc, pH 6.5, 1U/ml, 16 hrs/ 37 °C

chondroitinase ABC (Sigma): 0.5M Tris/0.5M NaCl pH 8.0, 1U/ml, 30 hours/ 37 °C

Typically, to 45 μ l (~150 μ g) of IMHV or whole brain P2 fraction (in 25 mM Tris pH 7.4) 5 μ l of the appropriate 10 times concentrated incubation buffer was added, containing the

desired amount of enzyme. The incubations were stopped by freezing or boiling in SDS-PAGE sample buffers.

5.13 Lectin blotting

The same process was repeated as described for the Western blotting, however, instead of incubating with monoclonal antibodies, the gels were incubated with biotinylated lectins (EY). *Ulex europaeus*, *anguilla anguilla* and *lotus tetragonolobus* were purchased as freeze dried proteins. A stock solution of 1 mg/ml was therefore prepared for each lectin in 0.015M PBS/0.15 M NaCl containing 0.1% sodium azide. Blots were incubated at 25 µg/ml final concentration for 2 hours at room temperature. Horseradish peroxidase conjugated avidin (Sigma) was used instead of secondary antibodies at 1 µg/blot concentration. Visualisation was by the insoluble peroxidase substrate DAB (3,3'-diaminobenzidine tetrahydrochloride buffered with urea/H₂O₂, Sigma).

6. Analytical approaches to glycan analysis

The objectives of any glycobiological analysis are twofold: to gain structural and functional information about the oligosaccharides. They both require unique technical and methodological approaches, however our knowledge of the glycan structures is far more advanced compared to the understanding of their biological function. This is due to the lack of suitable techniques and the typically low sample yields of subcellular fractionation procedures.

The non-linear sequence of oligosaccharides, as opposed to proteins and oligonucleotides, makes their structural analysis quite complex. The actual process of glycan analysis has to accommodate the special requirements of each individual sample, therefore in practice a unique selection of methods is often necessary for different samples. However, the general strategy typically involves the following steps:

- glycan release (chemical or enzymatic)
- glycan labelling (fluorescent or radioactive)
- glycan characterisation and fractionation (monosaccharide composition, hplc and gpc profiling)
- structural analysis of individual glycan components

Glycans can be released and labelled only with a limited number of methods. However, glycan characterisation, fractionation and structural analysis can be accomplished in a number of different ways, depending on the laboratory hardware, the sample amount and personal preference. This typically involves a monosaccharide compositional analysis and glycan fractionation based on charge and size. Structural information –at picomol sample levels- is typically obtained by sequential enzymatic digestions and/or by reagent array analysis (RAAM) method. These methods will be discussed in some detail next.

6.1 Glycan release

The analysis of N- or O-linked glycans requires their release from the glycoprotein. The most common methods are chemical and enzymatic deglycosylation.

Chemical release can be achieved in several different ways, including β -elimination under mild alkaline conditions to release O-linked glycans (Carlson 1968), hydrazine to release N-linked oligosaccharides (Bayard 1975), or lithium aluminium borohydride to release both N- and O-linked oligosaccharides (Likhoshesterov et al. 1990).

The O-glycosidic linkage is stable under alkaline conditions except when the linkage occurs between a sugar and an aglycon, which contains an activated hydrogen atom in β position of the anomeric linkage (Figure 6.1) This is the situation when mono- or oligosaccharides are linked to the serine or threonine residues of the proteins, therefore alkaline conditions are used for the selective release of O-linked glycans from glycoproteins. Linkages between hydroxylysine and hydroxyproline are stable in alkaline solutions.

The reaction conditions during mild alkaline conditions (0.05-0.1 M NaOH) would lead to the degradation of the released glycans by a “peeling-reaction”, therefore excess NaBH_4 is included to stabilise the reducing end of oligosaccharides.

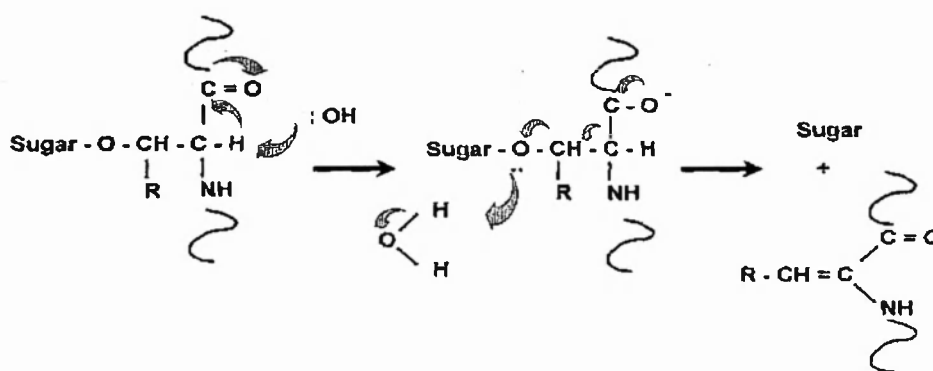


Figure 6.1. The mechanism of β -elimination during O-linked glycan release (from Spik 1995)

N-linked glycans are typically released from glycoproteins or glycopeptides by hydrazine at 100 °C for 8-14 hours. Hydrazine also causes the cleavage of peptide bonds and loss of

acetyl groups from N-acetylhexosamine residues. The free amino groups are therefore re-N-acetylated prior to the isolation of oligosaccharides. Under the standard conditions of 100 °C/14 hrs the O-linked glycans are also released but subsequently destroyed.

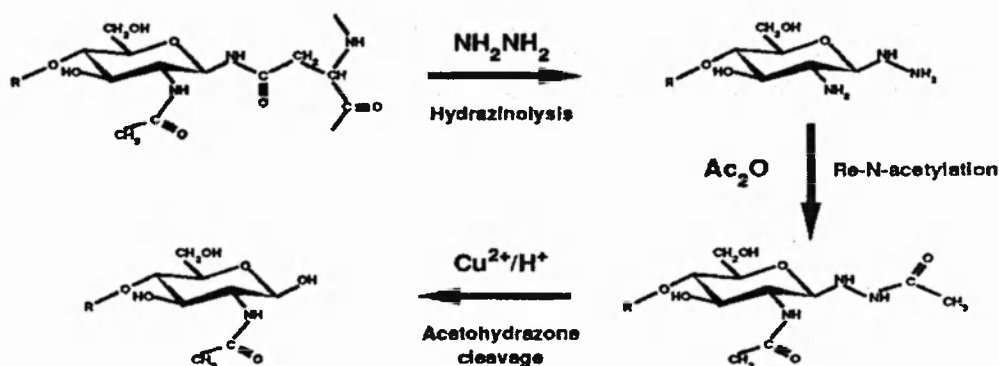


Figure 6.2. Hydrazinolysis of asparagine-linked oligosaccharides (from GlycoPrep 1000 Oxford GlycoSciences user manual).

Therefore the original procedure was further developed by Patel et al. (1993) to allow the simultaneous liberation of both classes of oligosaccharides. Moreover, the process has been automated offering three different release conditions suitable for N-only, O-only, or N+O-linked glycan release. The N+O mode has been used throughout the experiments presented in this thesis. Automated hydrazinolysis involves the following steps: (also see Figure 6.2):

- the actual hydrazinolysis, where the N- and O-glycosidic bonds are cleaved to release the glycan structures from the protein or peptide
- removal of the unreacted hydrazine
- chromatographic separation of the glycans from the peptide material and amino acid hydrazides
- re-N-acetylation of the glycans and removal of the ionic material by desalting
- finally the acetohydrazone derivatives are converted to unreduced glycans by the addition of catalytic amounts of Cu^{2+} ions

In addition to the chemical methods, enzymes are also used for the release of glycan from glycoproteins or glycopeptides (O'Neill 1996). These enzymes offer the advantage of preserving the intact protein. Moreover, they are the method of choice when the precise ratio of N-acetyl and N-glycolyl substituted sugars is of importance, because these groups

are destroyed during the hydrazinolysis step. Several enzymes are available with different specificities but compared to N-linked glycans, the enzymatic release of O-linked sugars is much more limited.

One of the disadvantages of the enzymatic release can be the steric hindrance due to bulkier glycan structures, to three-dimensional conformation of the protein, or due to the presence of certain sugar substituents, which can inhibit certain enzymes.

From the commercially available enzymes glycoamidase F (also known as PNGase F and N-glycanase) have been used during the experiments described in this thesis. This enzyme was first recognized by Plummer et al. (1984). Characterisation of its enzymatic activity indicated that the broad substrate specificity of this glycoamidase could make it useful in release of all classes of N-linked oligosaccharides including high-mannose, hybrid, complex sialylated and even sulphated structures up to and including tetraantennary structures. The only feature of an oligosaccharide that appears to prevent its release by PNGase F is the presence of an α 1-3 linked fucosyl residue on the reducing end N-acetylglucosamine, but this substituent tend to occur in plants.

The enzyme has been cloned and is commercially available as a recombinant protein from a large number of suppliers.

6.2 Fluorescent labelling

Labelling of the released oligosaccharides is necessary, because naturally occurring oligosaccharides do not possess an intrinsic biophysical property that could be exploited for their sensitive detection during the separation steps. Traditionally, radioactive labelling has been used, but the recently developed fluorescent dyes offering subpicomolar sensitivity became more popular.

There are several conditions that have to be fulfilled for an effective label: it should be detectable at low levels after HPLC and must be stable during separation, purification and structure determination (Hase 1994). Moreover, the labelling process must be quantitative

and non-selective without structural modification of the labelled glycan. Several dyes have been developed that fulfil the above criteria, and the choice of a particular dye depends on the method being used during the subsequent glycan analysis. The most common fluorophores used in glycan analysis are the 2-aminobenzamide (2-AB), 2-anthranilic acid (2-AA), 8-aminonaphthalene-1,3,6-trisulphonic acid (ANTS), 2-aminopyridine (2-AP) and 2-aminoacridone (AMAC). Some of the dyes are also negatively charged (e.g. ANTS) therefore glycans tagged with these dyes can be separated by electrophoresis (40-60%) and visualised by fluorescence monitoring. This is also known as the FACE™ technology.

Reductive amination is the principle of the chemical reaction between the dye and reducing oligosaccharide. The terminal reducing N-acetylglucosamine of the N-linked or the N-acetylgalactosamine of O-linked glycans exists in equilibrium between the ring-open and ring-closed conformation (Figure 6.3). The aldehyde group of the ring-open conformation (B) reacts with the primary amine group of the dye under acidic conditions to form a Schiff-base. This compound is relatively unstable and is stabilised by reduction with a mild reductant to form a secondary amine. 2-aminobenzamide has been used for the fluorescent tagging of chicken oligosaccharides throughout this thesis.

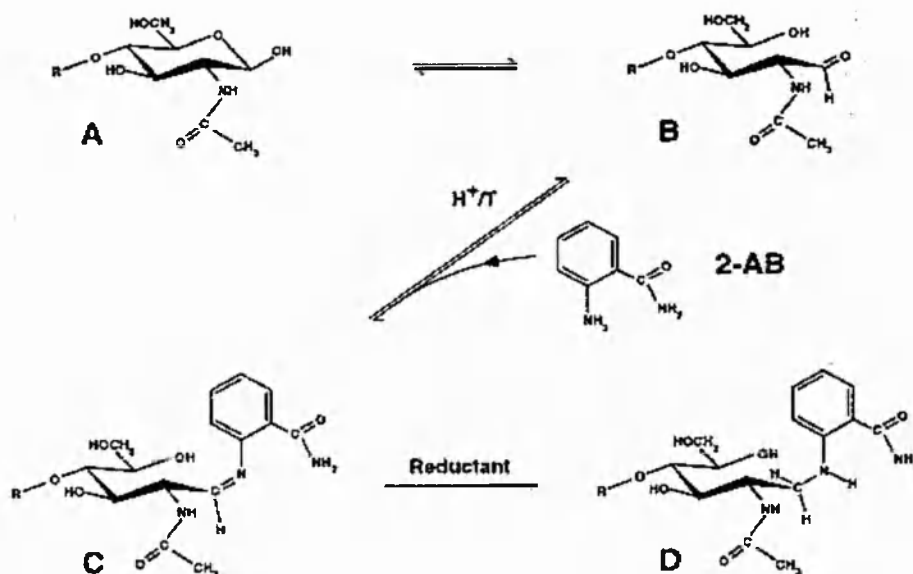


Figure 6.3. Fluorescent tagging of oligosaccharides with 2-aminobenzamide. (A) ring closed, (B) ring open conformation. R-rest of the oligosaccharide (from the 2-AB Signal Labeling Kit™, Oxford GlycoSciences)

The dye, 2-aminobenzamide, 2-AB, when conjugated to a glycan has the following fluorescent parameters: $\lambda_{\text{exc}} = 330 \text{ nm}$ and $\lambda_{\text{em}} = 420 \text{ nm}$, which allows sensitive (picomols) inline detection of conjugated glycans by a fluorescent detector.

6.3 *Monosaccharide compositional analysis*

Monosaccharide analysis can be very useful as a first step during glycan analysis. The composition and ratio of individual components may help to direct further analysis. For example, the absence of GalNAc may indicate the lack of mucin type glycans in the sample. The ratio of hexosamines, hexoses and deoxyhexoses may be an indication of the ratio of complex/hybrid vs. high-mannose structures.

The methodology currently available for monosaccharide analysis ranges from colorimetric assays to gas-liquid chromatography and mass spectrometry (Spik 1995, Rudd et al. 1997). A recent technical development in monosaccharide analysis was the introduction of pellicular* anion exchange resins, which have significantly improved the resolution of monosaccharides. The CarboPac PA-1 (Dionex) column used in our laboratory contains quaternary ammonium functional groups bonded to polymerised styrene/divinylbenzene (PS-DVB). During separation monosaccharides are completely ionised by the high pH of the elution buffer (typically sodium hydroxide, pH~13) and are eluted in order of their decreasing pK_{a} . The method is also known as high-pH anion exchange chromatography (HPAEC) of monosaccharides.

Another important aspect of monosaccharide (and oligosaccharide) analysis is the detection system, because it determines the minimum amount of sample necessary for analysis. As mentioned earlier, naturally occurring saccharides do not contain functional

* Pellicular resins are characterised by having a thin ion exchange copolymer film or latex, bound to the surface of inert micro-bead. The high specific surface area, low capacity and short diffusion paths of these resins are especially well suited for the separation of monosaccharides (Harland 1994).

groups that would allow their sensitive detection, and refractometric detectors are not sensitive enough for common analysis.

One approach to overcome this problem is the derivatisation of monosaccharides with fluorophores or chromophors, similarly to oligosaccharides, as mentioned above. However, with the recent introduction of pulsed electrochemical detection (PED) systems, this step can be omitted and monosaccharides directly detected in the effluent.

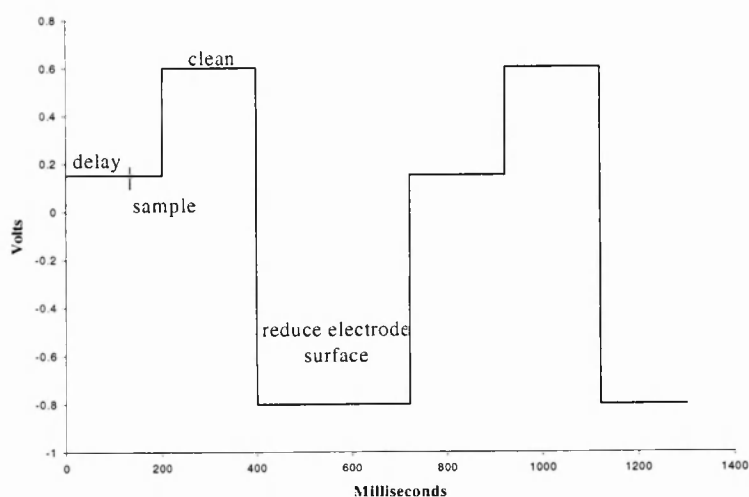


Figure 6.4. Changes in electrode potentials during the pulsed electrochemical cycle

The method is based on the electrochemical oxidation of analytes. A very small aliquot of the sample, which is in the form of oxyanions due to the high pH of the elution buffer, is oxidised on a noble metal electrode, which generates an electric signal. This oxidation current is then measured and constitutes the signal sent to the integrator/recorder. The noble metal is required to lower the oxidation barrier of non-aromatic poly-hydroxyl compounds. However, the electrode is fouled with the quickly decaying signal, therefore a high positive and negative potential is applied to “clean” the electrode. This pulsed sequence of potentials is illustrated on Figure 6.4. Some of the monosaccharides may not be completely ionised by the elution conditions sufficient for their detachment from the resin column. Because complete ionisation is necessary for the PAD detection, eluants are

mixed with a high concentration of sodium hydroxide solution (300 mM) just before entering the detector cell. This is called the post-column derivatisation.

6.4 High-performance liquid chromatography in glycan analysis

The next step after releasing and labelling a glycan pool is their separation into individual components, in order to characterise these components at the structural level. If monosaccharide analysis indicated the presence of charged residues (sialic acids, glucuronic acids) it may be useful to separate the total glycan pool on the basis of net negative charge by ion-exchange chromatography. The charged fractions can be individually pooled and further analysed. The resulting neutral structures after the removal of negatively charged residues can be separated according to their size (or hydrodynamic volume) or according to their overall hydrophilicity by gel-permeation chromatography or normal-phase HPLC, respectively.

Ion-exchange chromatography

Ion exchange chromatography separates individual components on the basis of their net negative charge. Weak-ion exchange chromatography is preferred, because in this mode the salt concentration is lower, and is therefore easier to remove. This is an important aspect in preparative mode, when individual fractions have to be pooled and desalted before further analysis.

GlycoSepC, the column used during this thesis, is a polymer-coated divinylbenzene matrix based column with weak anion-exchange groups (DEAE). The column is calibrated with sialic acid containing 2-AB labelled structures, derived from bovine fetuin. The structures are separated according to their charge, but multiple peaks may elute at a defined charged region reflecting the underlying structure and size of the sialylated oligosaccharide. Larger e.g. triantennary structures elute before smaller biantennary structures.

The elution buffer used with this column contains only low concentrations of volatile salts (ammonium formate) which can be easily removed by rotary evaporation under reduced pressure.

Normal-phase chromatography of carbohydrates

In normal phase, sugars are separated on the basis of their hydrophilicity. The hydroxyl groups of the sugar residues interact with the polar functional groups of the column, and are eluted with increasing salt concentrations.

Perhaps the major advantage of the HPLC technology is the relative speed of separation and its flexibility. The elution conditions can be easily adapted to the requirements of particular samples by adjusting the buffer composition, elution time and gradient applied. The technology has been developed into a reproducible, well documented and easy-to-use methodology, and is certainly one of the major recent advancements in the field of glycan analysis (Rudd 1997, Neue and Phoebe 1997).

Reversed-phase chromatography of carbohydrates

This type of chromatography separates oligosaccharides according to their overall hydrophobicity. This mode is particularly useful for separating structures that co-elute in normal phase. It is the extent of accessibility of hydrophobic regions on the oligosaccharide that governs the interaction with the C18 column, therefore the effect of linkage position is more pronounced in this mode for two similar structures, than during normal phase, where the hydrophilic interactions predominate. Reversed phase HPLC is often used to separate identical structures with and without bisecting GlcNAc.

6.5 Gel filtration chromatography

Gel-permeation chromatography (GPC, also called P4, gel filtration or size exclusion chromatography) separates molecules on the basis of their hydrodynamic volume, which is by definition the volume occupied by a molecule in a given solution (for example when water is the eluant). It is the function of the three-dimensional structure and the molecular

weight of the molecule. This particular separation method has been extensively used for glycan analysis since the late 50's, well before the development of HPLC techniques described above.

Glycans are separated in long columns (typically 0.5-2 meters) packed with porous acrylamide based material (although other stationary phases, like cross-linked methacrylate, cross-linked acrylamide and sulphonated styrene-divinylbenzene are also used [Edge 1992]). The pore size is an important factor during separation. The separated molecules penetrate into the pores, the rate and extent of penetration is the function of their hydrodynamic volume. As glycans pass through the column, an equilibrium is established between the molecules trapped inside the pores and in the column buffer. Smaller molecules can easily penetrate into the pores, and hence are slowed down compared to larger molecules during the separation process. By choosing the appropriate pore size, column dimensions, flow rate and temperature, high-resolution separation conditions suitable for naturally occurring oligosaccharides can be achieved.

If a mixture of glycans of known sizes is separated, a relationship between their retention volume and glycan size can be established. The relationship is typically defined by a third-order polynomial fit. This equation permits the size of an unknown structure to be determined from its elution position by extrapolation. Controlled hydrolysis of dextran (a polymer of glucose) results in a mixture of glucose oligomers with different degree of polymerisation. This mixture is used to calibrate the column, therefore glycans are described in terms of "glucose units" or P4GUs.

This calibration step makes P4 chromatography a universal method for neutral glycan analysis (charged glycans elute in the void volume of the column due to the interactions with the column packing material). The P4 glucose unit values of a large number of oligosaccharides are now available in public databases, similar to other glyco-analytical

databases^{*}. Because there are natural restrictions to the variability of glycan structures (see Biosynthesis of N-glycans, p. 30), the hydrodynamic volume -which is the reflection of the glycan structure- can be used to predict some structural information. By searching the databases for structures with P4GU values of the unknown glycan, oligosaccharides with similar or identical P4GU values can often be found. Therefore such predictions may help to direct and/or reduce the number of subsequent analytical steps.

6.6 Enzymatic sequencing of N-linked glycans

Enzymatic methods for glycan analysis are particularly useful, when only a low levels of sample material are available. This method is often suitable to provide full and unambiguous sequence information, perhaps the only limitation is the availability of different exoglycosidases.

The enzymes used for sequencing are exoglycosidases, which cleave nonreducing terminal monosaccharides in a highly specific manner. Typically the specificity is at the level of anomericity, linkage type, and even structural features like the branching environment.

In practice, sequencing involves the repeated digestion of the sample with a number of enzymes. The digestions can be monitored by any technique, which allows the precise comparison of the untreated and digested sample. The most common methods are gel-permeation chromatography on Bio-Gel P4, HPLC or mass spectrometry.

The choice of the enzymes is critical and is made on the basis of sample properties (mass or size) combined with the rules of N-linked biosynthetic processes and the fine specificities of the exoglycosidases. After each digestion, the samples are cleaned up (desalted and deproteinated) and analysed by one of the methods mentioned above (HPLC during the course of this work). The digested fractions separated by HPLC are pooled,

^{*} SUGABASE – an NMR database containing proton and carbon shift values of complex carbohydrates; “The Complex Carbohydrate Research Center Neural Networks” database contains mass spectra of partially methylated alditol acetate derivatives generated during carbohydrate methylation analysis.

desalted from HPLC buffer salts if required, and another digestion is carried out as long as no further digestion is possible or necessary (typically, the Man β 1-4GlcNAc β 1-4GlcNAc-2AB structure).

The drawbacks of the method are that the process is iterative and time-consuming, each digestion requires 16-18 hrs, the cleaning up and drying processes another few hours and the separation may take 3 or more hours. Interpreting the results may not always be straightforward or possible, especially when non-typical samples are analysed.

A further development in enzyme sequencing is the **reagent array analysis method**, or **RAAM** (Prime et al. 1997). It is principally different from the sequential strategy as it uses a *mixture* of exoglycosidases rather than single enzymes. The sample is divided into several equal aliquots, and each aliquot is digested with a different mixture of enzymes, called the enzyme array. Each mixture digests the sample glycan to a single product, the stop-point fragment, which the mixture is unable to digest further. The products of all incubations are combined and injected onto the RAAM 2000 GlycoSequencer.

The chromatogram (experimental signature) is evaluated by the RAAM's built-in software, which contains the chromatograms of all the theoretically possible digestion outcomes for all the theoretically possible N-linked glycan structures. By comparing the experimental chromatogram (signature) with the theoretically possible signatures, the best matches are identified. The "similarity" of the chromatograms is measured by the Kolmogorov-Smirnov statistic. On the basis of this statistic, a "match-quality" is assigned to each result. For example a value > 90% indicates a very good match quality, 80-90% is good quality and lower values indicate marginal match qualities. The degree of confidence attributed to a given match-quality has been empirically determined (Prime et al. 1997).

7. Glycoanalytical methods – practical details

7.1 *Automated hydrazinolysis*

GlycoPrep 1000 (Oxford GlycoSciences) has been used for the automatic release of N- and O-linked oligosaccharides from delipidated synaptic plasma membrane (SPM) glycoproteins. Cell membranes and organic cellular material do not interfere with the release. Samples were free from salts, transition metal ions, detergents and non-volatile solvents. Detergent extracted pure SPMs were washed 3 times in 70% ethanol and finally precipitated with absolute ethanol. The final precipitate was also rinsed with 2 washes of absolute alcohol. The pellet was then exhaustively lyophilised, 2 mg weighed out and used for glycan release in N+O mode (see also p. 74). All reagents were purchased from Oxford GlycoSciences.

7.2 *Fluorescent glycan labelling*

The glycan pool from the hydrazinolysis preparation (in a solution of dilute sodium acetate and acetic acid) was filtered through a 0.2µm Teflon filter and dried down by rotary evaporation. The dried pellet was fluorescently labelled with 2-aminobenzamide using the Signal Labeling Kit™ from Oxford GlycoSciences.

The dried glycan pool was dissolved in 10µl (instead of 5µl as recommended by the instructions) of a solution of 70% dimethylsulphoxide, 30% glacial acetic acid containing 0.25M 2-AB and 1.0 M sodium cyanoborohydride, and incubated for 2 hours at 65 °C. Glycans were then cleaned up on a GlycoSepS cartridge by adsorption to the hydrophilic filter in the presence of acetonitrile when water is the eluant.

The resulting 2-AB labelled material (representing the N- and O-linked glycan pool associated with the synaptic plasma membrane glycoproteins) was then dissolved in 200 µl pure water and used for further analysis.

7.3 Monosaccharide release

Hexoses, hexosamines, and deoxyhexoses were released by acid hydrolysis. 0.5 mg of glycoprotein or ~100 pmol 2-AB labelled glycan was hydrolysed in eppendorf tubes for 4 hours at 100 °C in 6M trifluoroacetic acid (Sigma). The hydrolysate was then dried down in SpeedVac and reconstituted in pure water. An aliquot of this solution was analysed by HPAEC as will be described next. When monosaccharides were released directly from glycoproteins, the Amino-Pac Guard column (Dionex, 4 x 50 mm) was used.

Sialic acids were released by mild acid hydrolysis. 0.5 mg glycoprotein was hydrolysed in 0.1M HCl for 1 hour at 80 °C. The sample was then dried down, dissolved in pure water, and an aliquot analysed by HPAEC using separation conditions suitable for sialic acid separation.

7.4 High-pH anion exchange chromatography of monosaccharides on CarboPac PA1

HPAEC was performed on a Dionex workstation equipped with a Dionex Gradient pump, an eluant-degass module, a Gilson sample injector (Model 231), a Gilson Dilutor 401 and Dionex ED 40 electrochemical detector with gold working electrode.

Monosaccharides released by TFA hydrolysis were dried down, reconstituted in pure water and an aliquot analysed on CarboPac PA1 (4x250 mm, Dionex) equipped with a guard column (4x50 mm).

Buffer **A** was 18mM NaOH, buffer **B** was 200 mM NaOH and buffer **C** was 300 mM NaOH made up fresh using 50% (w/vol.) concentrated NaOH stock solution (BDH). All buffers were degassed for 15-20 minutes before the addition of appropriate volume of concentrated NaOH, and for 5 more minutes afterwards.

Table 7.1: Monosaccharide analysis on CarboPac PA1

Time (min.)	flow rate (ml/min)	%A	%B	%C (postcolumn, at constant 0.3 ml/min flow rate)
0	1	100	0	100
20	1	100	0	100
21	1	0	100	100
31	1	0	100	100
32	1	100	0	100
52	1	100	0	100

Standard mixture of fucose, galactosamine, glucosamine, galactose, glucose and mannose was separated before each unknown sample set (1 nanomol each, Dionex), which enabled the identification and quantitation of unknown samples.

7.5 HPAEC of sialic acids on CarboPac PA1

Sialic acids released by mild acid hydrolysis were separated on CarboPac PA1 equipped with a guard column. Buffer **A** was degassed pure water, buffer **B** was 200 mM NaOH and buffer **C** was 500 mM NaOAc. The following gradient conditions were used:

Table 7.2: Conditions applied during sialic acid separation on CarboPac PA1

Time (min.)	flow rate (ml/min)	%A	%B	%C
0	1	40	50	10
5	1	40	50	10
25	1	14	50	36
30	1	14	50	36
32	1	40	50	10
52	1	40	50	10

N-acetylneuraminic acid and N-glycolylneuraminic acid stock solutions (1mg/ml each) were prepared by dissolving the contents of vials of each monosaccharide (Sigma) in appropriate volume of pure water to give the desired final concentration. The sialic acids were not dried down before solubilization, therefore due to the possible water content -as a result of the hygroscopic properties of these sugars- the stock solution concentration may not have been absolute.

7.6 HPAEC of mannose-6-phosphate (test of alkaline phosphatase)

During the analysis of the charged glycan fractions, the presence of phosphorylated structures was tested by alkaline phosphatase (AP) treatment (E.coli, type III, Sigma). In order to test the enzyme activity before SPM glycan analysis, mannose-6-phosphate was digested with AP and the product analysed by HPAEC-PED.

Alkaline phosphatase treatment

20 µg of mannose-6-phosphate (Sigma) was digested with 2U of alkaline phosphatase in 20 µl 100 mM Tris-HCl buffer, pH 8 for 2 hrs at 37°C. In parallel, an aliquot of the mannose-6-phosphate was incubated under the same conditions but without the enzyme (blank-digest). Digests were analysed on CarboPac PA1 without prior desalting. The results (Figure 7.1, Figure 7.2) have confirmed the activity of the enzyme.

Table 7.3: Conditions used for mannose and mannose-6-phosphate separation on CarboPac PA1

Time (min.)	flow rate (ml/min)	%A (H ₂ O)	%B (200 mM NaOH)	%C (500 mM NaOAc)
0	1	30	50	20
20	1	10	50	40
25	1	10	50	40
27	1	30	50	20
40	1	30	50	20

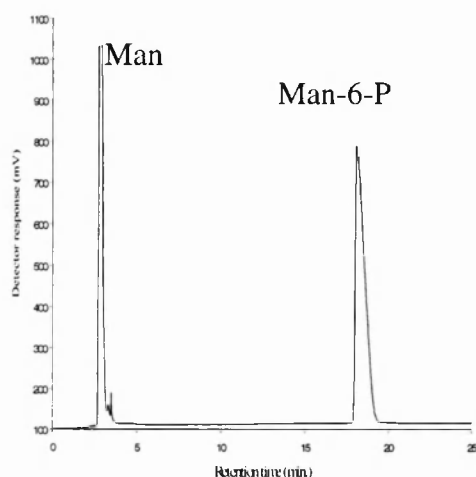
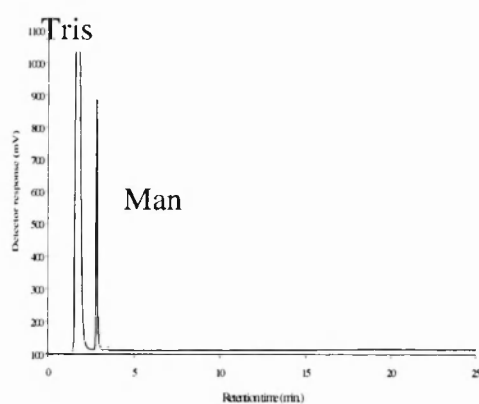
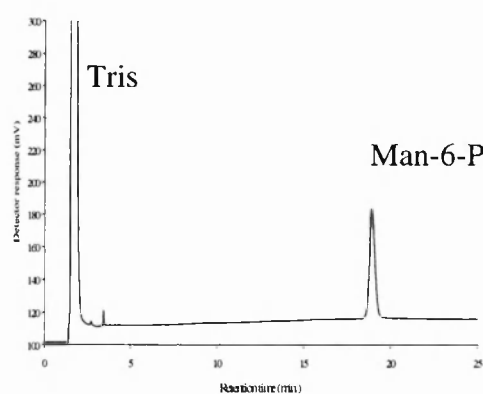


Figure 7.1: Mannose (2.9 min) and mannose-6-phosphate (18.1 min) in water.



a



b

Figure 7.2: a.) mannose-6-phosphate successfully converted to mannose eluting at 2.9 min by alkaline phosphatase b.) blank-digest. Note the large peak eluting at 1.9 minutes is due to Tris-HCl used as enzyme buffer.

7.7 Ion exchange chromatography on MonoQ HR 55

Similarly to the dephosphorylation reaction, the desulphation procedure was first tested on well-characterised heparin fragments. These sulphated and desulphated dimers were analysed by ion exchange HPLC on MonoQ (Pharmacia) using the following separation conditions:

Table 7.4: Separation conditions for heparin disaccharides on MonoQ HR 55

Time (min.)	Flow rate (ml/min)	%A (H ₂ O)	%B (1M NaCl)
0	1	100	0
45	1	0	100
47	1	100	0
60	1	100	0

The samples were monitored by UV detection at 232 and 214 nm. MonoQ HR 55 is made from Sephadex, therefore it is kept in 20% ethanol, to prevent bacterial growth.

The following heparin disaccharides were purchased from Sigma and mixed in equal amounts to give a mixture of standard heparin disaccharides:

Δ UA-2S-GlcNS-6S (disaccharide I-S)

$\Delta^{4,5}$ -uronic acid-2-sulphate-(β 1-4)-glucosamine-N,6-disulphate

Δ UA-GlcNS-6S (disaccharide II-S)

$\Delta^{4,5}$ -uronic acid-(β 1-4)-glucosamine-N,6-disulphate

Δ UA-2S-GlcNS (disaccharide III-S)

$\Delta^{4,5}$ -uronic acid-2-sulphate-(β 1-4)-glucosamine-N-sulphate

Δ UA-GlcNS (disaccharide IV-S)

$\Delta^{4,5}$ -uronic acid-(β 1-4)-glucosamine-N-sulphate

Δ UA-GlcNAc6S (disaccharide II-A)

$\Delta^{4,5}$ -uronic acid-(β 1-4)-N-acetylglucosamine-,6-sulphate

Δ UA-2S-GlcNAc (disaccharide III-A)

$\Delta^{4,5}$ -uronic acid-2-sulphate-(β 1-4)-N-acetylglucosamine

Δ UA-GlcNAc (disaccharide IV-A)

$\Delta^{4,5}$ -uronic acid-(β 1-4)-N-acetylglucosamine

A stock solution of each disaccharide (5 mg/ml) was prepared in water. A 20 µl aliquot was taken from each stock solution and mixed. 25 µl from the final mixture was injected and separated on MonoQ under conditions described in Table 7.4. p. 90.

Desulphation

Samples (an aliquot of the heparin disaccharide mixture or 2-AB labelled chicken brain glycans) were dried down under a gentle stream of nitrogen in Pierce's glass vessels. Desulphation was allowed to proceed for 18 hrs at room temperature in 500 µl of dry 50mM methanolic-HCl (made up by diluting 0.5M anhydrous methanolic-HCl with dry methanol). The reaction was stopped by transferring the solution from the vessel to a tube suitable for evaporation under reduced pressure. Residual HCl was removed by evaporation from 500 µl of water and the oligosaccharides re-N-acetylated by the addition of 500 µl saturated sodium bicarbonate solution and two aliquots of 20µl of acetic anhydride (Fluka) separated by a 10 min interval. The reaction was allowed to proceed at room temperature for 50 min, then the oligosaccharides were desalted by passage through 500µl of AG50 x 12 (H⁺ form), eluted with water and concentrated by evaporation. The desulphated disaccharides were analysed on MonoQ (Figure 7.3), 2-AB labelled glycans on GlycoSepC.

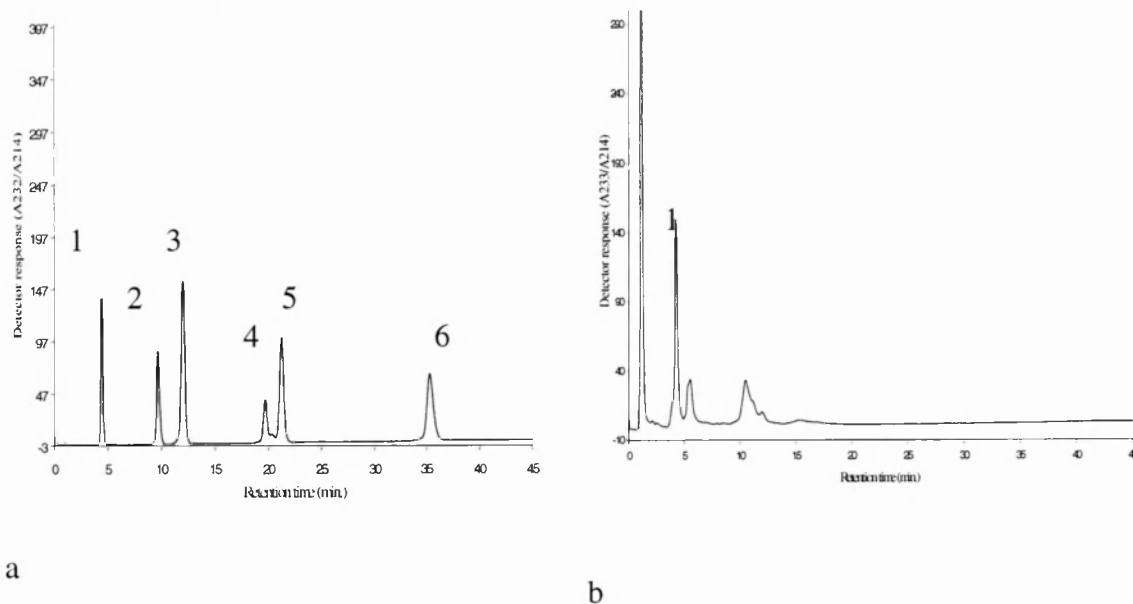


Figure 7.3: Heparin sulphate mixture before (a) and after (b) mild methanolysis and separated on MonoQ. The identity of the individual peaks (in order of increasing retention time):

Δ UA-GlcNAc, Δ UA-GlcNS, Δ UA-2S-GlcNAc, Δ UA-GlcNS6S, Δ UA-2S-GlcNS, Δ UA-2S-GlcNS6S

(b) peak 1 represents the Δ UA-GlcNAc neutral disaccharide, the end-product of desulphation.

7.8 HPLC- High performance liquid chromatography

Oligosaccharides were analysed by three different modes (normal, weak-ion exchange and reversed phase) using different HPLC columns and a Waters workstation.

The system consisted of a Waters 717 Plus Autosampler, a Waters 600S Controller, 626 Pump and an in-line-degasser module. GlycoSepN and GlycoSepC HPLC columns were obtained from Oxford GlycoSciences.

The 2-AB labelled oligosaccharides were monitored in-line in the effluent by a F1000 Fluorescent Spectrophotometer (Merck-Hitachi) at $\lambda_{exc}=330$ nm and $\lambda_{em}=420$ nm. The spectrophotometer was connected to a desktop personal computer (Digital 466) via a SATIN Bus/LACE module (Waters). Chromatograms were obtained and processed using the Millennium 2000™ HPLC analytical software.

Weak ion-exchange chromatography on GlycoSepC

Charged 2-AB-labelled glycans were separated by HPLC on GlycoSepC, which is a polymer-coated divinylbenzene matrix based column with weak anion-exchange groups (DEAE). Buffer A was 20% acetonitrile in water, buffer B was 20% acetonitrile in 200 mM ammonium formate pH 4.5. The gradient details are listed in Table 7.5.

Table 7.5: HPLC conditions used with GlycoSepC for charged glycan analysis

Time (min)	flow rate (ml/min)	%A	%B
0	0.3	100	0
5	0.3	100	0
40	0.3	0	100
45	0.3	0	100
45.2	0.3	100	0
50	0.3	100	0

2-AB labelled fetuin (containing mono- di- tri- and tetrasialylated structures) was used to calibrate the column before each sample set, and used as an external reference for unknown charged glycans.

Normal phase HPLC on GlycoSepN

For normal-phase separation a method developed by Guile et al. (1996) was used in combination with the GlycoSepN column, which is silica based with amide functional groups. The pH of the solvents is adjusted to 4.4, which is the pK of amide, to minimise ion-exchange effects when charged glycans are present in the analyte. Samples adsorb to the column at high concentrations of organic solvent (acetonitrile) and are eluted with an increasing aqueous gradient. As the elution position was found to be sensitive to small changes in temperature by (Takehi 1993), the column was equilibrated to constant temperature (30.6 °C) throughout the runs.

Solvent A was 50 mM ammonium formate, pH 4.4 and solvent B was acetonitrile. The gradient conditions for normal phase HPLC were:

Table 7.6: Normal phase HPLC on GlycoSepN - short gradient

Time (min.)	flow rate (ml/min.)	%A	%B	Curve
0	0.4	35	65	linear
72	0.4	53	47	linear
75	0.4	100	0	linear
77	1.0	100	0	linear
92	1.0	100	0	linear
95	1.0	35	65	linear
100	0.4	35	65	linear

Table 7.7: Normal phase HPLC on GlycoSepN - long gradient

Time (min.)	flow rate (ml/min.)	%A	%B	Curve
0	0.4	20	80	linear
132	0.4	53	47	linear
135	0.4	100	0	linear
137	1.0	100	0	linear
142	1.0	100	0	linear
145	1.0	20	80	linear
180	0.4	20	80	linear

The columns were calibrated with a mixture of 2-AB labelled dextran oligomers before each sample set. Elution times were then plotted against glucose units. The 3rd order polynomial equation fitting this plot was calculated by the Grafit™ software and used to calculate the NPGU values of unknown samples (Figure 7.4).

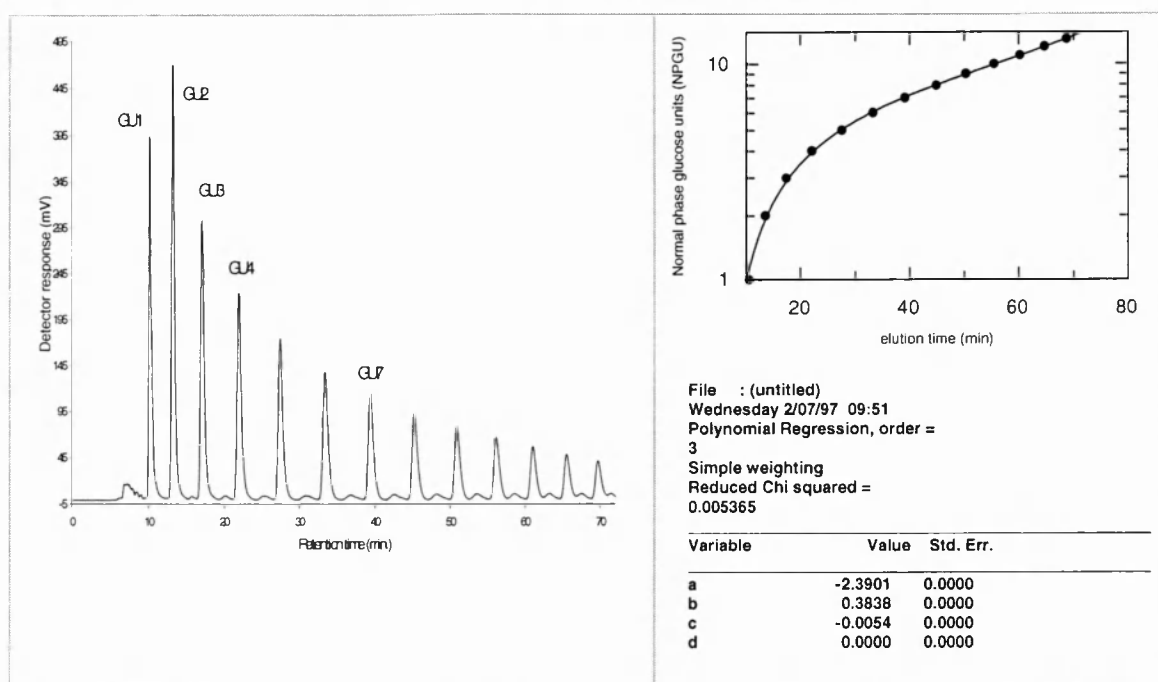


Figure 7.4. An example of the normal-phase calibration procedure using the short gradient. On GlycoSepN. The polynomial equation is used to calculate the NPGU values of unknown samples on the basis of their retention times on GlycoSepN.

7.9 Gel-permeation chromatography

Gel permeation chromatography (GPC) was routinely performed on the RAAM GlycoSequencer's sizing column (10 mm x 480 mm). Separation conditions recommended by the manufacturers have been used for optimal results.

During gel filtration chromatography of chicken brain derived 2-AB labeled neutral and neutralised glycans the "high-resolution" flow profile was selected, which is a gradient with the following components:

phase A with a flow rate of 35 $\mu\text{l}/\text{min}$ for 11ml,

phase B with an increased 160 $\mu\text{l}/\text{min}$ flow rate for 27 ml.

phase C at a flow rate 160 $\mu\text{l}/\text{min}$ for 7 ml.

The total volume was 45 ml.

During RAAM digest analysis the flow profile was at a constant flow rate of 60 $\mu\text{l}/\text{min}$ for 12 hours 30 minutes.

The GlycoSequencer has a built-in fluorescence detector and a refractometer, with detection limit 12 picomol/peak for fluorescently labelled samples and 1 μ g/peak for unlabelled sugars.

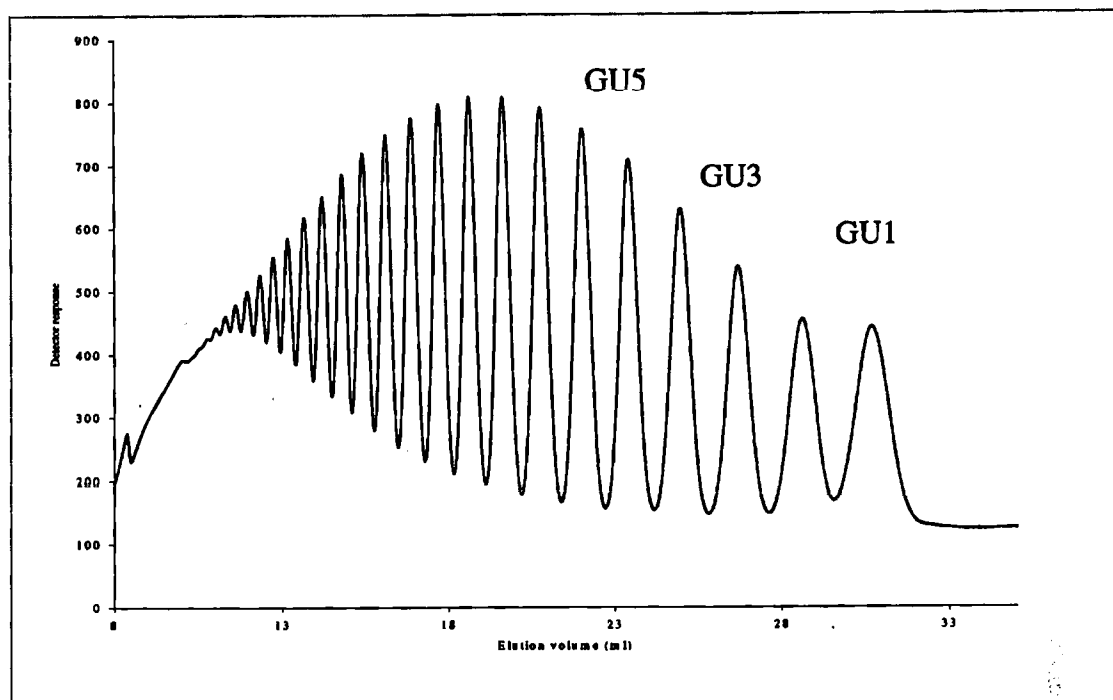


Figure 7.5. An example of a dextran hydrolysate separated on P4 and used for column calibration

Dextran hydrolysate was used for automatic column calibration as an internal standard (detected by the in-line refractometer); co-injected with the samples, except when samples were pooled for further analysis Figure 7.5.

7.10 Enzyme digestion conditions used during sequential sequencing

The following digestion conditions have been used throughout the experiments described in the following chapters. All enzymes were from the Oxford GlycoSciences (unless otherwise stated), including the enzyme buffers.

Endo- β -galactosidase (Bacteriodes fragilis)

125 mU/ml

50 mM sodium acetate pH 5.8 containing 250 μ g/ml BSA and 1 mM NaCl *α -Mannosidase (Jack bean)*

50 U/ml (10-100 U/ml) enzyme concentration

100 mM sodium acetate buffer pH 5.0 containing 2 mM Zn^{2+} *β -N-acetylhexosaminidase (Streptococcus (Diplococcus) pneumoniae)*8 mU/ml (for β 1,2 specificity)

100 mU/ml (nonspecific)

100 mM sodium citrate/phosphate buffer, pH 6.0

Sialidase (Newcastle Disease Virus, Hitchner B1 Strain)

0.2 U/ml

50 mM sodium acetate pH 5.5

Sialidase (Arthrobacter ureafaciens)

1-2 U/ml

100 mM sodium acetate pH 5.0

 α 1-3/4 fucosidase (Almond meal)

0.2 U/ml

50 mM sodium acetate pH 5.0

 α 1-2/3/4/6 fucosidase (Bovine kidney)

0.2 U/ml

100 mM sodium citrate pH 6.0

 α 1-2 fucosidase (Xanthomonas manihotis) (BioLab)

0.5U/ml

50 mM sodium citrate buffer (pH 6.0) 20 hours / 37 °C

7.11 Desalting after enzyme digestions

Glycans digested with exoglycosidases were desalted and deproteinated on a tandem of Dowex AG1 (acetate form) over AG50 (triethylamine form) using 150-200 μ l bed volume which was covered with a 30 μ l layer of C18 resin. In some cases the same columns were

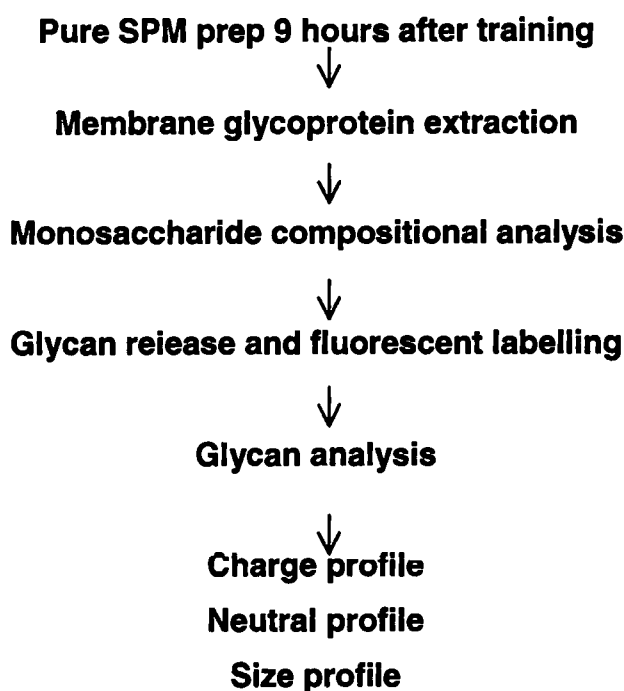
loaded into fine eppendorf pipette tips using 10-15 μ l of each resin (“micro-column cleanup method”). Oligosaccharides were eluted with 3 column volumes of pure water.

Alternatively, digests were deproteinated using a 0.45 μ m cellulose nitrate (CN) Pro-Spin Micro™ centrifugal filters. The samples were applied to the filter, the proteins left to adsorb for 30-60 minutes at room temperature and the samples recovered by centrifugation three times with 50 μ l 5% acetonitrile.

When charged glycoproteins were analysed, carbon cartridges (GlycoCleanH, Oxford GlycoSciences) were used according to the manufacturer’s instructions.

8. Overview of the pure SPM sample set glycan analysis

In the following chapters the glycosylation profile of chicken SPM glycoproteins will be described. Pure synaptic plasma membrane glycoproteins were prepared from the forebrains of chickens trained on a passive avoidance learning task and from age-matched, same-hatch quiet animals 9 hours after training. The overview of the experiments is illustrated below, they have been performed in parallel for MeA trained and quiet control chickens:



The results are organised into five chapters. Chapter 8 describes the glycosylation profile of the pure SPMs from the forebrain, and compares this profile between the trained and quiet control chicks. In chapter 9 the same analysis is performed, however on different samples, derived from a small brain region, the intermedial medial hyperstriatum ventrale (IMHV). Chapter 10 deals with the glycan profile of dark-hatched chicken SPMs. Structural information about the most abundant complex-type glycans is presented in chapter 11. And finally, chapter 12 explores the applicability of a recent analytical method for the glycan profiling of chicken SPM glycoproteins separated by PAGE.

8.1 Monosaccharide compositional analysis

Trifluoroacetic acid release of monosaccharides

In order to get a preliminary estimate of the glycan structures present, (e.g. GalNAc typically indicates the presence of O-linked glycans, the ratio of Man to GlcNAc and/or Gal reflects the presence or absence of hybrid/complex type structures), the monosaccharide composition of SPM glycoproteins was determined. As there is no method for a single-step quantitative release of all the naturally occurring monosaccharides, different conditions optimised for certain monosaccharides are used. The reason for this is in the nature of chemical bonds between the single sugar components, and the acid stability of the released monosaccharides. As none of the methods can release all the component monosaccharides quantitatively, the monosaccharide analysis –esp. when complex mixtures of whole tissue glycans are analysed- should be interpreted as a general guideline about the approximate composition of the glycan mixture. In case of single purified proteins, or tissues derived from specific developmental/disease stages, however such a monosaccharide analysis can be of important diagnostic value.

Due to limited sample availability TFA hydrolysis alone was used to estimate hexose, fucose and hexosamines even though hexosamine release was not expected to be quantitative. Both trained and quiet samples were analysed, however due to the non-quantitative nature of monosaccharide release direct comparison of the two experimental groups was not possible. Sialic acids are destroyed by this method, therefore they had to be released under milder conditions using 0.1M HCl.

Monosaccharide compositional analysis was also performed on the *glycan* pool. This was necessary since the pure SPMs were prepared by sucrose density gradient centrifugation, and although several washing steps were included, glucose contamination was still observed when *glycoproteins* were used as a starting material for monosaccharide

hydrolysis. For convenience, 2-AB labelled glycans were used, as 2-aminobenzamide does not seem to interfere with the monosaccharide profile.

Individual peaks were identified and quantitated using the monosaccharide calibration curve. Results are expressed as nanomol/mg of dry weight and as a ratio to glucosamine (GlcN) as an internal standard.

High pH anion exchange chromatography (HPAEC) on CarboPac PA-1 column

The column was first equilibrated for sufficient time under the starting gradient condition until a stable baseline was obtained. Usually, this was achieved by doing a “dummy” run, when water was injected. Afterwards 10 μ l (containing 1 nanomol of each component) of standard monosaccharide mixture was injected.

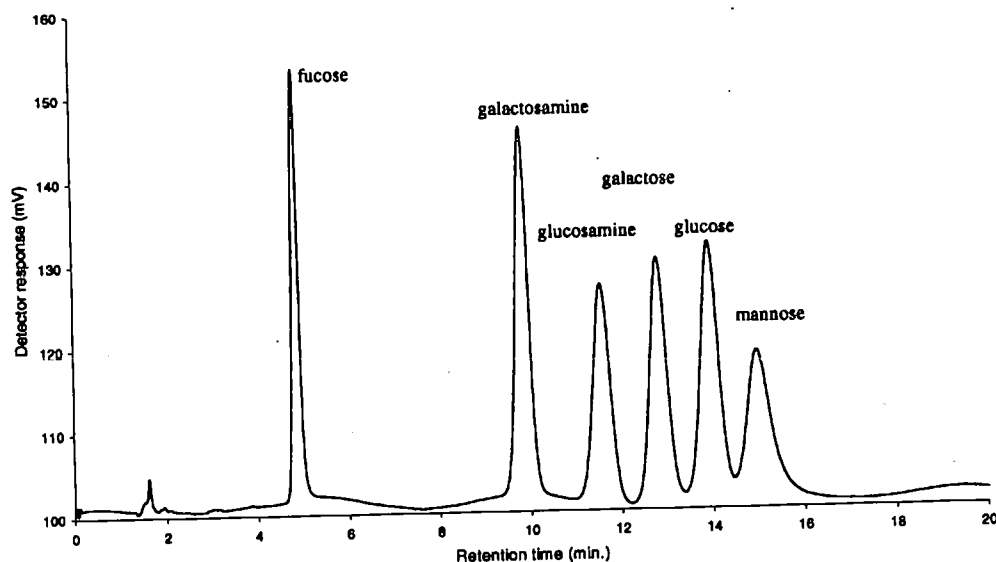


Figure 8.1. Standard monosaccharide mixture (1 nmol each) was separated and the response factor (nanomol/area) used for quantitating unknown samples.

Table 8.1. Standard monosaccharide peak areas corresponding to 1 nanomol

Monosaccharide	Ret. time (min)	Area/1 nanomol
Fuc	4.83	553686
GalN	9.83	901558
GlcN	11.56	676058
Gal	12.80	762873
Glc	13.93	858115
Man	14.96	639686

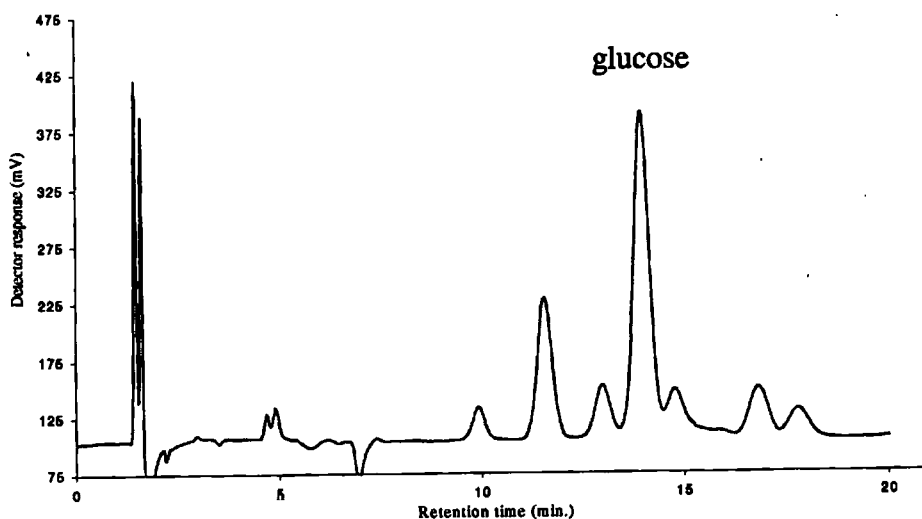


Figure 8.2. Monosaccharide profile from pure SPM *glycoproteins*. The prominent glucose peak originating from the membrane fractionation procedure prevented the quantification of individual monosaccharides.

The TFA acid hydrolysate of the 2-AB labelled glycan pool showed no obvious contamination from glucose. Peak areas were used for monosaccharide quantitation using the monosaccharide standard responses (Table 8.1). The overlay chromatograms of monosaccharides from the MeA trained and quiet control groups are shown on Figure 8.3.

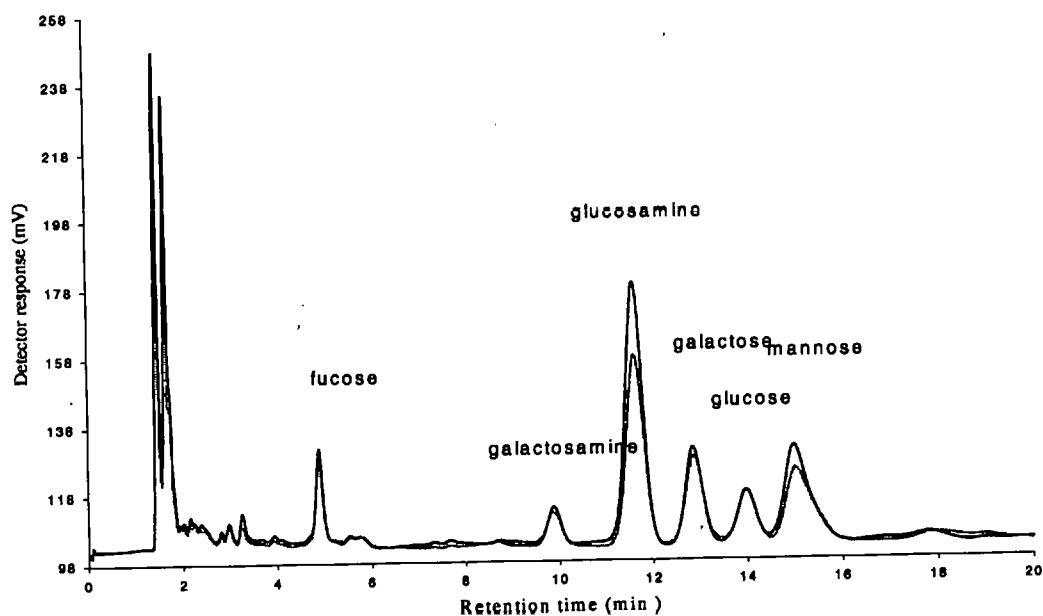


Figure 8.3. Monosaccharide composition of the 2-AB labeled glycan pool from MeA trained and quiet chickens. Gray line – MeA trained; black line- quiet control.

Table 8.2. Monosaccharide compositional analysis. Values for adult rat brain SPMs are also shown for comparison.

Monosaccharide	MeA trained		Quiet birds		Zanetta (1977)
	nanomol/ mg protein	Ratio to GlcN	Nanomol/ mg of protein	Ratio to GlcN	Ratio to GlcN
Fucose	6	0.25	5.9	0.27	0.29
Galactosamine	2.7	0.11	2.3	0.11	0.12
Glucosamine	23.9	1	21.7	1	1
Galactose	10.2	0.42	8.9	0.41	0.59
Mannose	15.1	0.63	15.7	0.72	1.1
Glucose	5.7	0.23	4.8	0.22	--

Sialic acid analysis

Sialic acids were released from the glycoproteins by mild acid hydrolysis using 0.1M HCl, which releases most of the sialic acids with some degradation of N- and O-acyl derivatives.

Glucose would not interfere with the separation, because different chromatographic conditions apply to sialic acid analysis.

Sialic acid standards were prepared by mixing equal amounts of N-acetylneuraminic acid and N-glycolylneuraminic acid solutions (1mg/ml each) to give a final solution of 0.5 mg/ml. The stock solutions were prepared by dissolving the contents of vials (N-acetylneuraminic acid and N-glycolylneuraminic acid obtained from Sigma) in an appropriate volume of GlycoPure water to give 1mg/ml solutions. The concentration of these solutions is not absolute, because of the hygroscopic properties of these acids.

Sialic acids were separated on CarboPac PA1 column equipped with a guard column (4 x 50 mm). The details of the solutions and gradients applied are described on p.86.

1µg of the standard mixture was injected and the elution position and detector response used for the identification and approximate quantification of neuraminic acids in the unknown sample solutions.

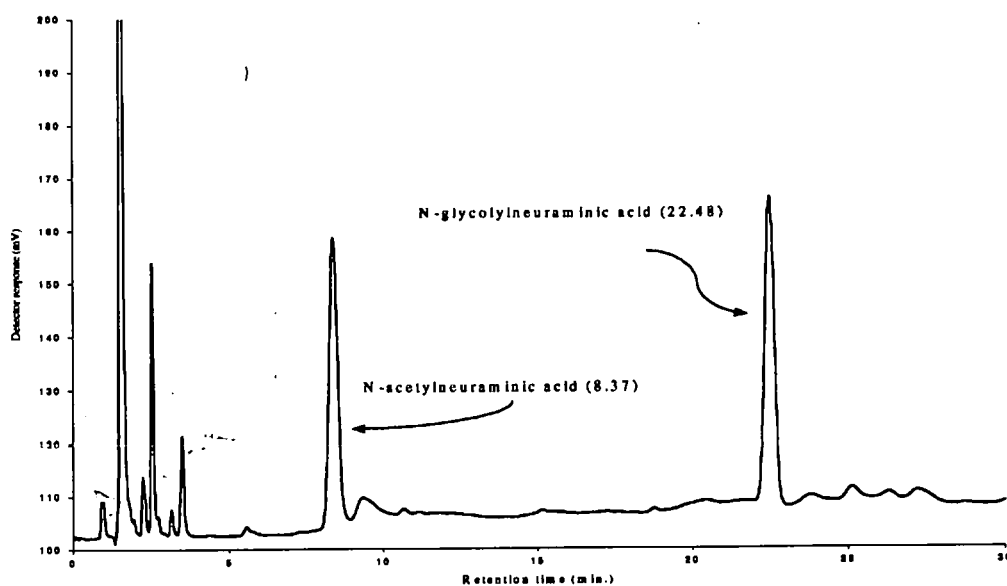


Figure 8.4. The mixture of N-acetyl- and N-glycolylneuraminic acids was separated on CarboPac PA1 coupled to a PED detector. The elution positions and detector response factor (area/µg) was used for the analysis of unknown samples.

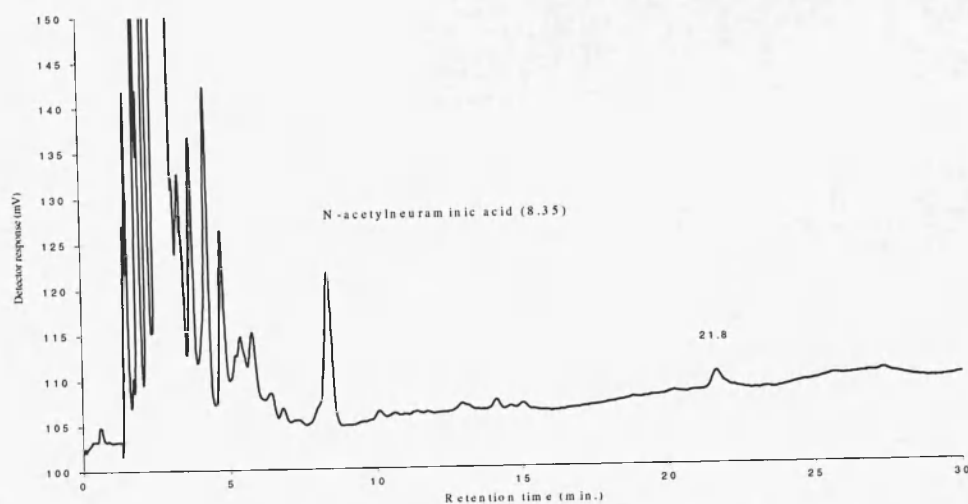


Figure 8.5. Mild acid hydrolysate of the SPM glycoprotein fraction analysed on CarboPac PA1 under identical conditions as used for the sialic acid standards.

N-acetylneuraminic acid was detected, however the peak eluting at 21.8 minutes could not be identified. The results expressed as μg sialic acid per mg of protein are shown in Table 8.3.

Table 8.3. Sialic acid content of the detergent extracted material from pure SPMs. Protein content of the dry starting material was taken into account during calculations.

Sample	Peak area	nanomol NeuAc/mg protein	μg sialic acid/mg protein
MeA	271279	6.18	2.04
Quiet	351191	5.43	1.78

Discussion

Monosaccharide analysis detected 6 monosaccharide components, namely fucose, galactosamine, glucosamine, galactose glucose and mannose.

The ratio of glucosamine to mannose (Table 8.2) indicates the presence of significant amount of complex/hybrid structures.

From the sialic acids N-acetylneuraminic acid was identified. Other minor peaks were noted as well, however their identity was not further investigated.

One of the main reasons for not characterising the monosaccharide composition in more detail (i.e. different conditions, like 4M HCl, for the more quantitative release of amino-sugars (Takehi 1993) was that the sample was derived from the forebrain tissue, and not from single glycoproteins. Therefore comparison of tissue monosaccharide profiles from forebrains when only minute changes are expected is not feasible. This is in part reflected in the very similar values obtained for the two samples. The other reason was the very limited sample material. Pure synaptosomal fraction preparations in general have low yields.

The data are in good agreement with other published results (Zanetta 1977, Krusius 1978b), however minor differences could be due to the very different technical approach and to the fact, that in those studies adult rat SPMs were analysed.

As a next step, glycans were characterised according to their charge, hydrophilic properties and hydrodynamic volume.

8.2 Charged glycan analysis of pure SPM glycoprotein glycans

When analysing unknown oligosaccharide samples, several strategic approaches can be taken. All of these methods lead to the simplification of the starting material by separating them into a group of components with a common property (like charge, affinity to specific lectins, size, hydrophobicity etc.). In this instance, due to the laboratory hardware, the purpose of the study, and the nature of the sample (tissue glycan pool), the first separation was on the basis of negative charge.

The charged oligosaccharide profile was obtained by weak-ion exchange chromatography on GlycoSepC (p.93). Before each sample set, the column was calibrated with a well-characterised glycan mixture (2-AB labelled fetuin) carrying variable number of sialic acids. Although the retention time is the function of the actual conformation of the glycan structures (the number of antennae, α 2-3 vs. α 2-6 linkage of the sialic acid etc.), it can be used as a “marker” to identify tentative mono- di- tri- and tetrasialylated structures.

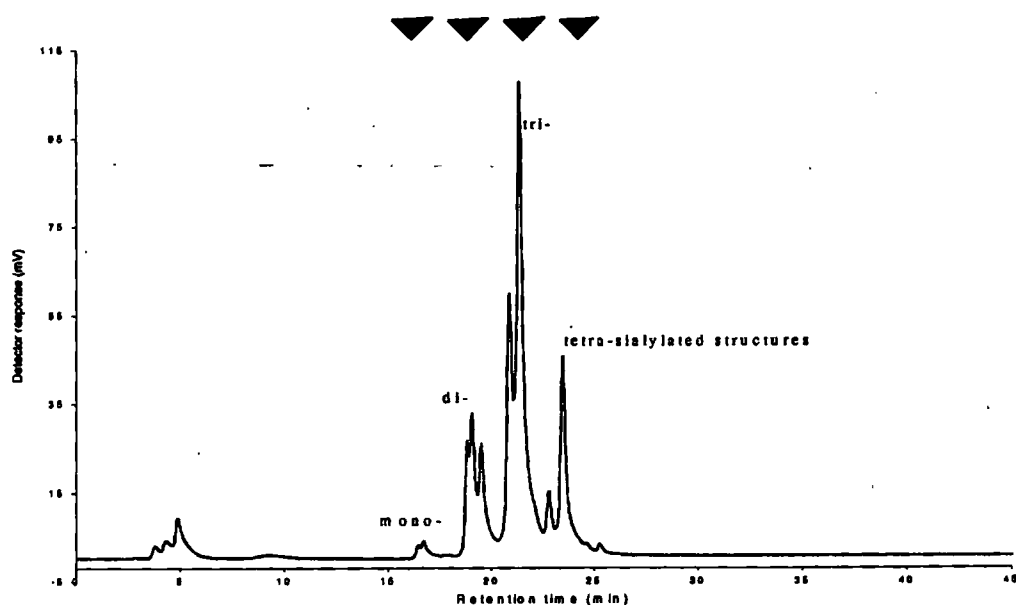


Figure 8.6. Fluorescently labelled charged glycans from bovine fetuin used for GlycoSepC calibration

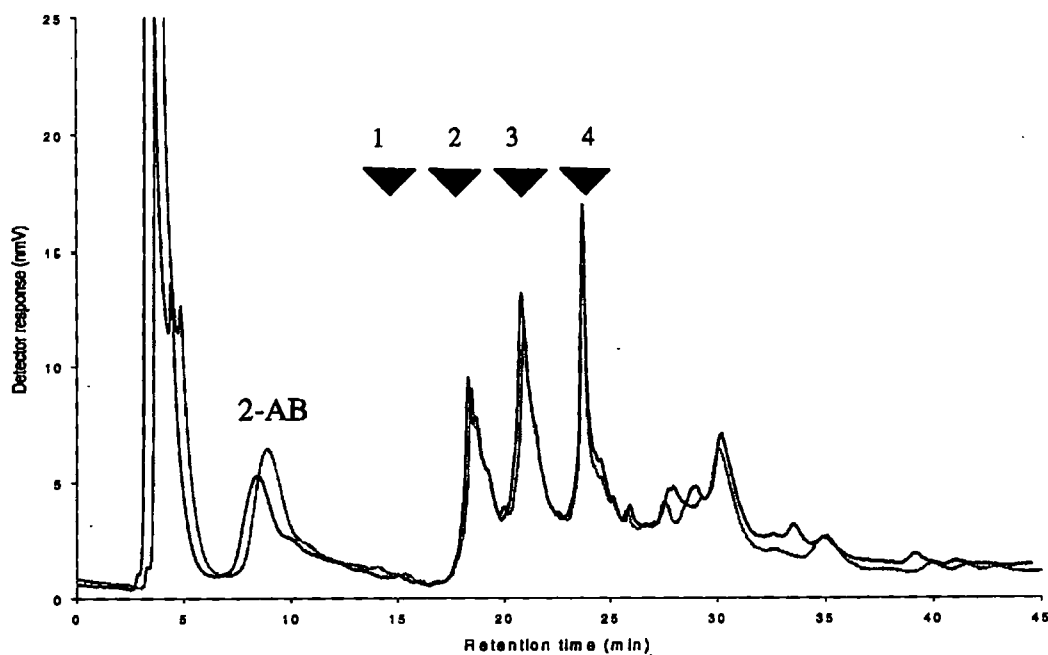


Figure 8.7. Total charged glycan profile from trained (grey) and quiet (black line) animals. The approximate elution positions of di-, tri- and tetrasialylated structures are indicated by the arrows.

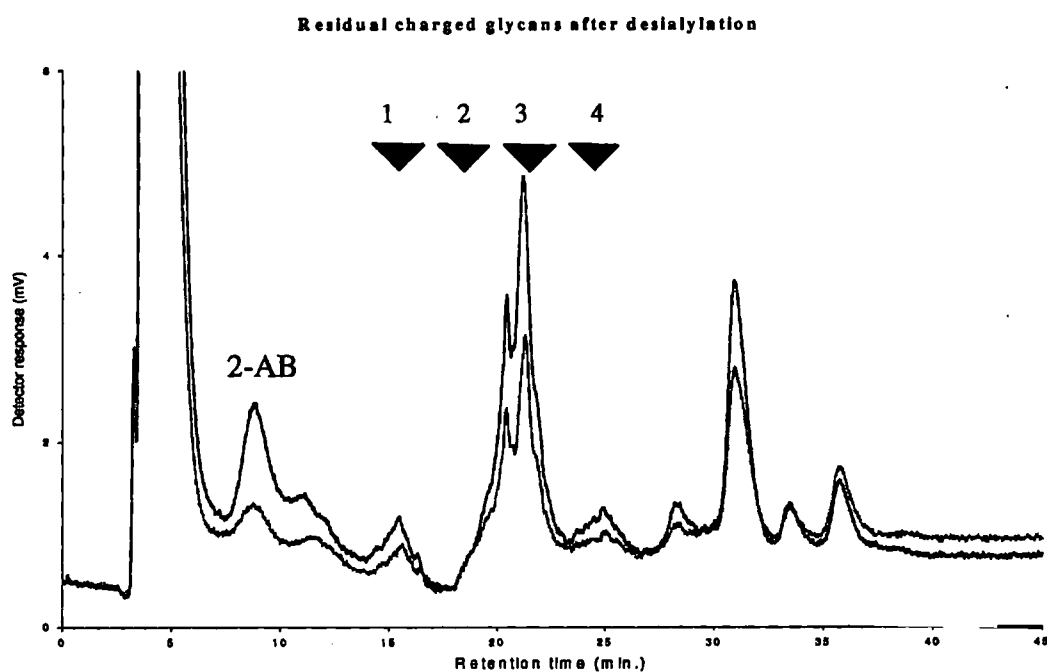


Figure 8.8. After complete desialylation residual charged material was still observed accounting for 15% of the total glycan pool.

No difference in the naturally charged profile was noted. Although sialic acid is the most common negatively charged residue in glycoprotein associated glycans, other residues, like

phosphate and/or sulphate may also be present. To examine this possibility, the total glycan pool was treated with sialidase (*Arthrobacter ureafaciens*), which removes α 2-3,6,8 linked sialic acids, the digest was cleaned up and at the same time desalted on GlycoCleanH (OGS) columns (not on ion-exchange columns), and finally rerun on GlycoSepC under identical conditions.

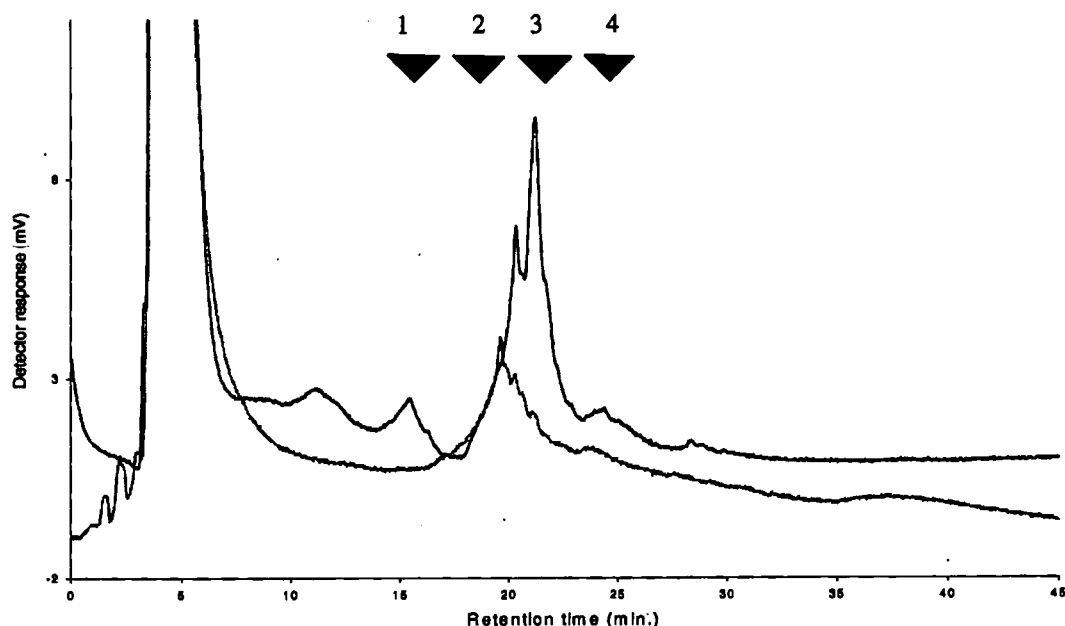


Figure 8.9. After desialylation and dephosphorylation a few anionic species were still present.

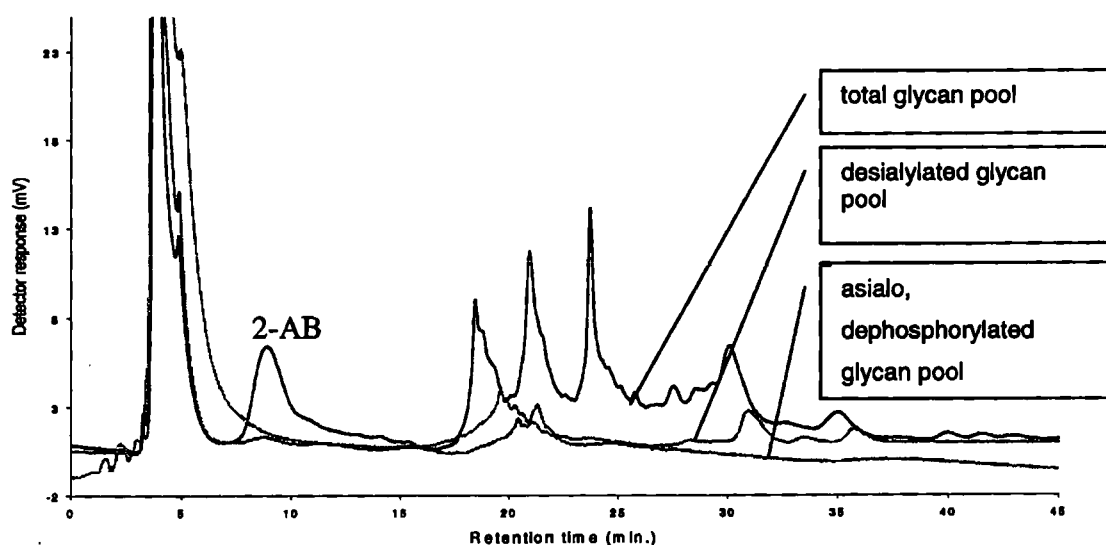


Figure 8.10. A summary overlay chart of the total charged glycan pool, desialylated and finally dephosphorylated glycan pool. Neutral glycans elute between 3 and 6 minutes.

Alkaline phosphatase (AP) will cleave all terminal phosphate residues in monoester linkages, but it does not cleave phosphate in phosphodiester linkages. The enzyme activity and specificity was first tested on mannose-6-phosphate as described on p.88.

Treatment of the desialylated glycan pool with AP lead to the disappearance of charged peaks eluting at positions after 23 minutes, as can be seen on the summary overlay chart (Figure 8.10). Therefore those peaks are most probably phosphorylated structures.

However, some residual anionic glycans were still present even after desialylation and dephosphorylation eluting around 25 minutes (Figure 8.10). Therefore the asialo-, dephosphorylated glycan pool was subjected to mild methanolysis, in order to selectively cleave sulphate esters. After desalting an aliquot of the desulphated sample was analysed on GlycoSepC. No charge material was observed, indicating that the residual material after alkaline phosphatase treatment could be due to sulphate substituted oligosaccharides.

Table 8.4. Summary of the charged and neutral glycan distribution on the basis of peak areas corresponding to charged and neutral structures.

Glycan pool	MeA treated		Quiet control	
	Neutral glycans	Charged glycans	Neutral glycans	Charged glycans
Total charged glycans	43%	57%	39%	61%
Desialylated glycans	85%	15%	85%	15%
After further dephosphorylation	94%	6%	91%	9%

Peak areas have been used to determine the relative contribution of individual anionic species to the net charge glycan pool. Samples after each enzymatic treatment were cleaned up on carbon columns to remove the enzyme and buffer salts, therefore the peak eluting early during the chromatogram should represent the neutral glycan species, with minor (if any) salt content.

As can be seen from Table 8.4, sialic acids accounted for 42% of all the charged material, phosphate 9% and sulphate and other minor components for 6% in the case of MeA trained sample. Similar values were obtained for quiet samples, 46% sialic acid, 9% phosphate and 6% sulphate and other minor components.

Discussion

In the first set of experiments the charged glycan profile was analysed. Comparison of the chromatograms from MeA trained and quiet chickens showed no difference.

Changes in polysialic acid synthesis after certain learning tasks in the rat have been previously reported in the literature (Welzl et al. 1996, Becker et al. 1996). However it is not known, where (or whether) polysialic acid would elute from the GlycoSepC column under the conditions described, therefore the separation conditions applied may not be suitable for this highly charged structure. One of the most straightforward methods for PSA analysis would have been to digest an aliquot of the glycan pool with endoN neuraminidase (which only acts on polymers of sialic acid, while other terminal, single sialic acids are not digested Reuter and Schauer 1994), and to compare the profiles before and after digestion.

The presence of phosphate and possibly sulphate in the charged fraction indicates that charged residues other than sialic acid contribute to the net charge of the synaptic plasma membrane glycans. The identities of these glycan structures have not been investigated in detail.

Phosphorylated glycans might be due to phosphorylated oligomannose oligosaccharides. Such phosphorylated structures have not yet been described on glycoproteins from the synaptic plasma membrane associated glycoproteins. Margolis and Margolis (1989) reported phosphorylated oligomannosidic glycans on the epidermal growth factor receptor present in the cell membrane of cultured cells. Whether similar glycans are present on membrane glycoproteins *in vivo* is not yet known.

Sulphate is known to occur in brain glycoconjugates, typically on N-linked glycans as N-acetylglucosamine-6-sulphate and galactose-6-sulphate (Guile et al. 1996). The best characterised sulphated epitope is the HNK-1, present on a number of neuronal cell adhesion molecules.

Other experiments are required in order to characterise the nature of sulphated and phosphorylated glycans. As only one type of analysis was performed for each type of anionic structure, further experiments, like metabolic labelling with radiolabeled phosphate and/or sulphate should be performed to confirm these preliminary results.

Detailed structural analysis of these relatively minor charged components would require larger amounts of starting materials.

8.3 Neutral glycan profile

In this set of experiments the neutral and neutralised glycan profile of SPM glycoproteins was characterised. Glycans were desialylated by neuraminidase treatment, dephosphorylated by alkaline phosphatase digestion and desulphated by methanolysis. Residual charged material was removed by anion-exchange on AG 1 (acetate form).

The neutral and neutralised glycans were analysed by size exclusion chromatography (SEC) on Bio-Gel P4 and normal phase high-pressure liquid chromatography on GlycoSepN (p.94). Glycans from trained and quiet birds were separated and compared under identical conditions.

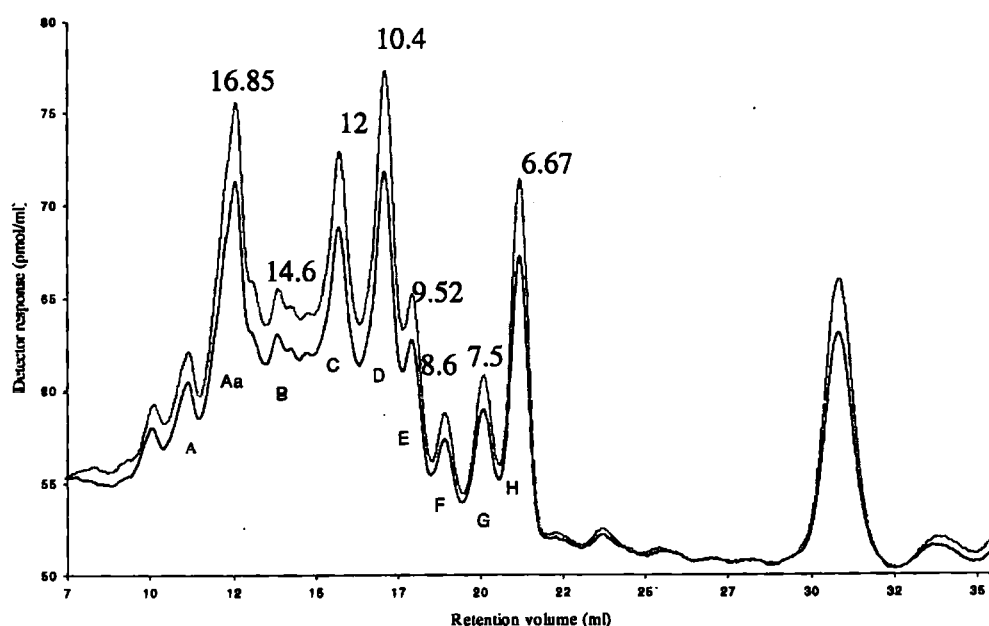


Figure 8.11. Neutral glycan pool from MeA trained and quiet chickens. No difference was noted between the two profiles.

The first neutral P4 profile was characterised by 8 predominant peaks Figure 8.11, their properties and relative abundance are summarised in Table 8.5.

Table 8.5. Glucose unit values and relative peak areas of P4 peaks. As can be seen, high-mannose structures (shadow) account for approx. 35% the total N-linked glycan pool, as peak D may contain other components as well.

Peak	Retention vol. (ml)	P4 GUs	Rel. area %
A	9.89	~20	12
Aa	12.05	16.85	18.9
B	13.36	14.58	13.9
C	15.18	12	16.9
D	16.56	10.4	16.1
E	17.4	9.52	5.6
F	18.39	8.58	3.5
G	19.58	7.55	3.4
H	20.68	6.67	9.7

In order to characterise the structures derived from the chicken brain SPMs, the complex glycan databases have been searched using the Eve software (RAAM GlycoSequencer, Oxford GlycoSciences). Peaks D, E, F, G and H had elution positions corresponding to high-mannose structures with 9,8,7,6 and 5 mannose residues, respectively. Therefore these peaks were pooled and digested with jack bean mannosidase, which cleaves terminal $\alpha(1,2/3/6)\text{Man}$ linkages. As can be seen on Figure 8.12., α -mannosidase treatment converted peaks E, F, G, H and the majority of peak D to a small structure eluting at 3.12 P4GU. This structure is almost certainly $\text{Man}\beta 1\text{-4GlcNAc}\beta 1\text{-4GlcNAc-2AB}$. Therefore structures E, F, G and H represent isomers of Man 8,7,6, and Man5. Peak D in addition to Man9 contains other component(s), which are not sensitive to jack bean mannosidase digestion.

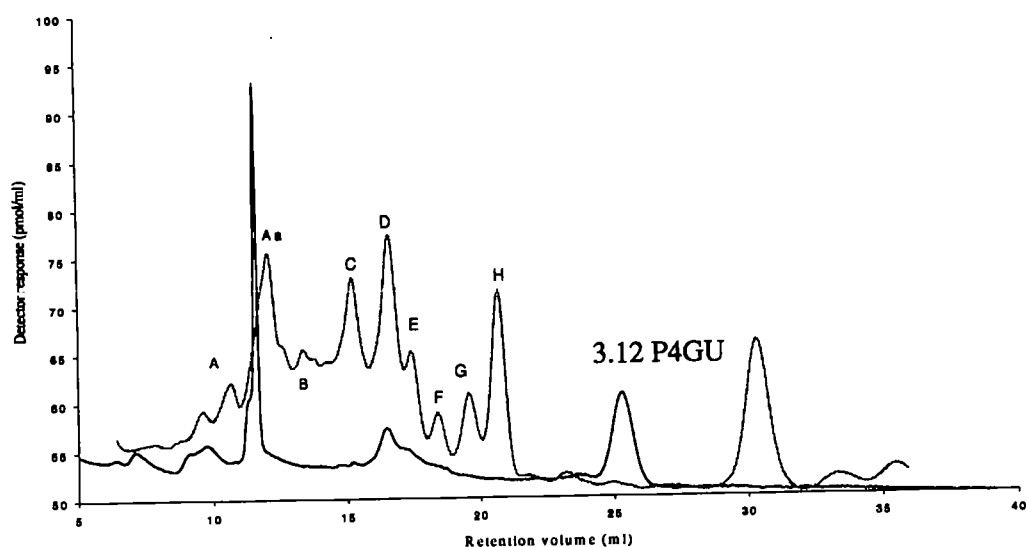


Figure 8.12. Peaks D,E,F,G and H were pooled (grey line) and digested with jack bean mannosidase (black line). Some of the peak D components were resistant to mannosidase digestion.

Due to the characteristics of the gel-sizing column, individual peaks may contain several different glycan structures, because different structures may have the same hydrodynamic volume. Therefore fine details may be lost when similar but not necessarily identical samples are compared. A more detailed resolution is achieved by normal phase HPLC. Moreover, this method has been successfully used for the comparison of IgG glycans derived from healthy individuals and patients with rheumatoid arthritis (Guile et al. 1996). Therefore it was expected, that qualitative differences between the neutral glycan profiles of the experimental groups -if present- should be detected by this method.

The chromatographic conditions were according to Guile et al. (1996). An aliquot of the *total* glycan pool has been separated first on GlycoSepN using the “short gradient” (p.94). The overlay chromatogram is shown on Figure 8.13. The “noise” at the 0-25 min. region is probably due to charged glycans, O-linked glycans and possible impurities, as the samples were not passed through an ion-exchange column prior to injection.

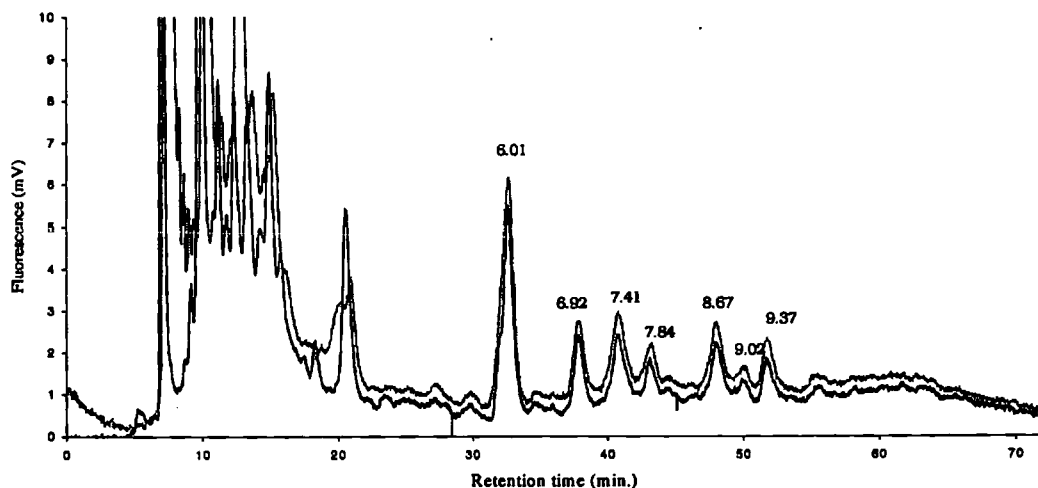


Figure 8.13. Neutral glycan pool on GlycoSepN (normal phase HPLC). Grey line: MeA trained, black line: Quiet control.

No difference between the naturally neutral glycans (eluting between 25-72 min.) was noted.

Search for α 1-2 fucosylated structures

Fucosylated structures were expected to be present on the basis of monosaccharide analysis, and most importantly on the basis of 2-deoxygalactose studies in chicks and rats (p.24). In order to identify the α 1-2 fucosylated structures an aliquot of the 2-AB neutral and neutralised glycan pool (from the MeA trained group) was digested with an α 1-2 linked fucose specific fucosidase from *Xanthomonas manihotis* (p. 97). Surprisingly, after separating the digests, no peaks were found to be sensitive to this enzyme (Figure 8.14). No 2-AB labelled glycans were bound to Ulex-I affinity lectin column either (data not shown).

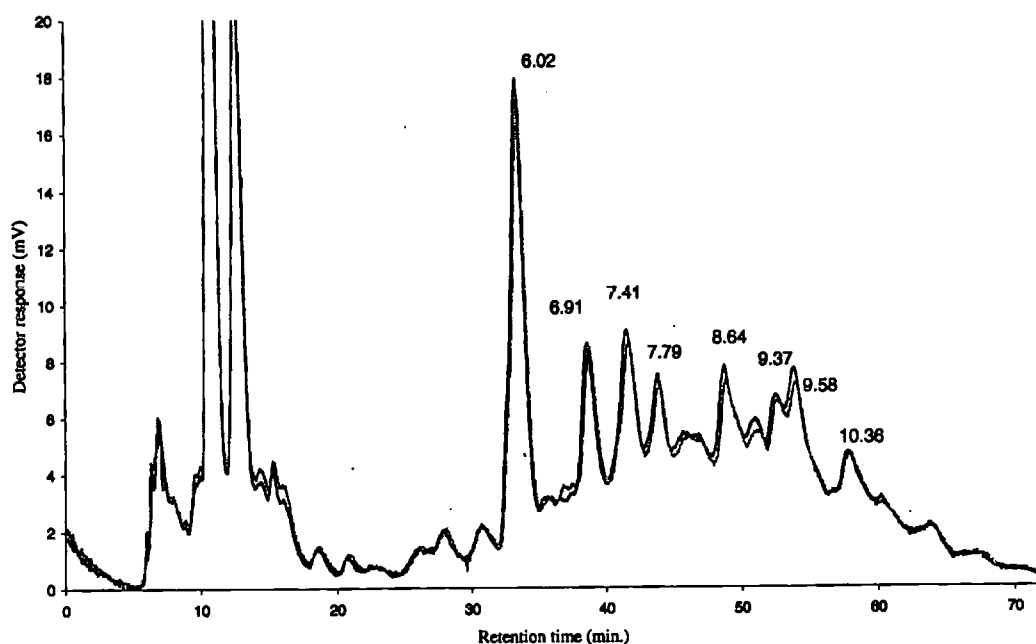


Figure 8.14. α 1-2 specific fucosidase digests of the neutral glycan pool. No α 1-2 fucosylated structures were detected. Digest (grey) and blank digest (black) are overlayed. Note the appearance of novel peaks possibly originating from the charged glycan pool after neutralization (peaks 9.58 and 10.36).

Simplifying the complexity of chromatographic profiles

The total neutral glycan pool seemed quite simple after P4 with only 8-9 predominant peaks. From the P4 glycan analysis it was known that approximately 35% of the N-linked glycans were high-mannose structures, which are possibly the dominant structures on GlycoSepN as well, judging from the HPLC GU values of the predominant peaks. In order to get an insight into the “underlying” complex structures, an aliquot of the mannosidase digested glycan pool was separated on GlycoSepN. A very complex chromatogram was obtained (Figure 8.15), the small glycans eluting between 4-6 NPGUs are possibly mannosidase sensitive hybrid structures.

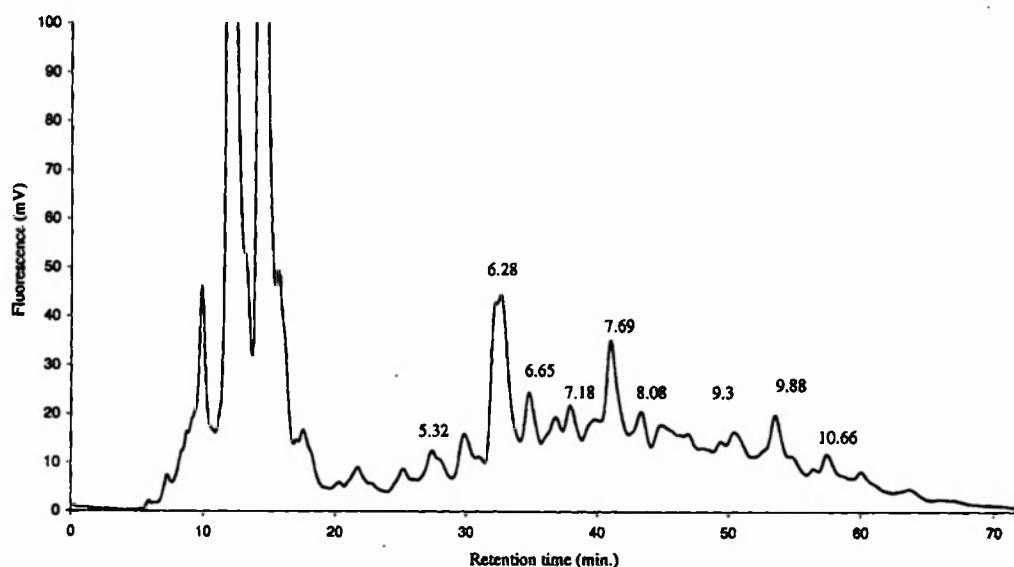


Figure 8.15. Neutral and neutralised glycan profile after mannosidase digestion on GlycoSepN.

In order to characterise the individual structures in more detail, fractions containing individual P4 peaks were pooled and analysed on GlycoSepN. Although this was time consuming, the complexity of the glycan profile required as detailed separation as was technically achievable, and moreover, the starting material had its P4 GU value characterised, which was useful during subsequent structure identification.

Individual P4 peaks

Individual P4 peaks were pooled and separated on GlycoSepN (Figure 8.16). As can be seen, a superior resolution was achieved by this additional preparative step, when compared to the total glycan pool on GlycoSepN.

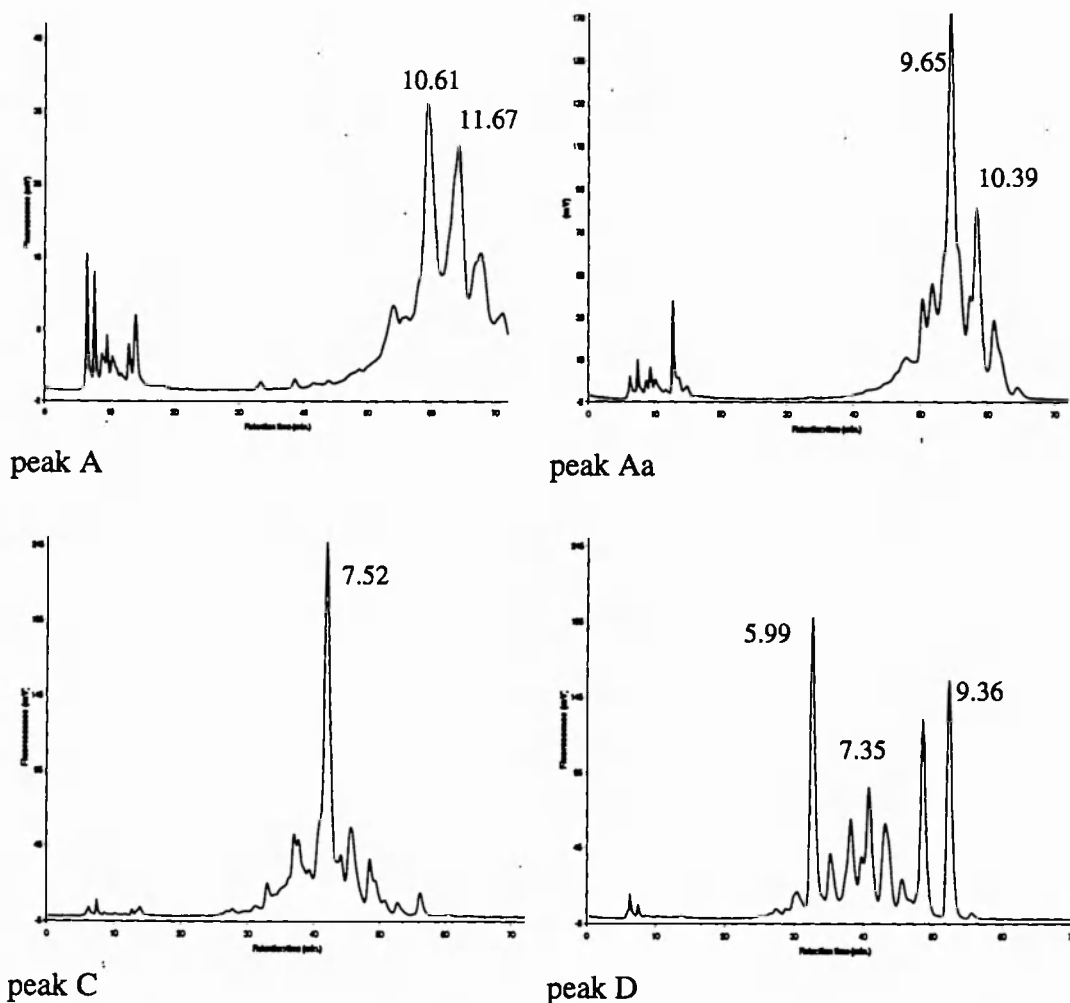


Figure 8.16. Individual peaks from the size-exclusion chromatography (Figure 8.11, p.113) have been pooled and separated on GlycoSepN (normal phase HPLC). Each individual peak is composed of several different structures coeluting on P4. Peak B is not shown.

Unfortunately the experimental groups could not be compared at the level of individual P4 peaks, because there was insufficient material to do the analysis. Although no learning related difference was evident when comparing the *total* glycan pools, the possibility that the individual P4 components may have had different GlycoSepN profiles could not be ruled out.

Discussion

In this set of experiments the neutral and neutralised glycans were characterised and compared. The first type of chromatography applied (P4-GPC) did not detect differences between the sample groups. However it helped to identify over 30% of the synaptic plasma

membrane associated N-linked glycan population, i.e. high-mannose structures with 5,6,7,8, and 9 mannose residues.

The absence of α 1-2 fucosylated structures may indicate that these structures are only minor components. However, as only one digestion, using standard recommended enzyme concentrations has been performed, it cannot be ruled out at the moment that the α 1-2 fucosylated glycans may be resistant to digestion under these conditions (although the enzyme was found to be active at this concentration on other compounds containing α 1-2 linked fucose, Dr S. Martin, personal comm.). It has been noted by Chen et al. (1998) that some of the α 1-3 fucosylated structures were resistant to almond meal fucosidase digestion at the recommended standard enzyme concentrations, and that removal of these residues could only be achieved with significantly higher enzyme concentrations.

The high proportion of oligomannosidic structures (>30%) at the synaptic plasma membrane fraction is intriguing. The function of this class of N-linked oligosaccharides is not well understood, esp. in the nervous system (also see p.55). Except for the well-known example of targeting the lysosomal enzymes to lysosomes via the phosphomannosyl-receptor, very few other interactions mediated by oligomannosidic structures have been described. The observations that high-mannose oligosaccharide structures drastically inhibit neurite outgrowth of cultured cerebellar neurons (Horstkorte 1993), and that N-CAM contains a lectin-like domain with affinity for high-mannose structures all indicate an important role for this structure. The second type of chromatography was based on the hydrophilic interactions between the separated glycans and the HPLC column. In this mode a more detailed separation was obtained – as compared to GPC-, however no difference between the experimental groups could be noted (Figure 8.13, p.116.).

Although the glycan profile of the nervous tissue associated glycans was not known before the analysis, the extreme complexity is not surprising or unexpected. As a consequence of this fact, however, the analytical methods had to be refined by combining different

chromatographic techniques and sample fractionation. This resulted not only in better separation, but also in dilution of the sample, and in order to stay within the boundaries of detection limit (1-10 picomols) sample groups had to be pooled to obtain some structural details.

9. Introduction to IMHV glycan analysis

The previous set of experiments (monosaccharide analysis, charged glycan and neutral glycan profile from the forebrains) did not detect any difference between the two groups of chickens. It is known from previous results from this laboratory that the biochemical changes are localised to relatively small brain regions, the intermediate hyperstriatum ventrale (IMHV) and the lobus parolfactorius (LPO). Moreover these changes are lateralised, i.e. the two hemispheres are involved to different degrees. Therefore it was suggested, that if changes occur in these small brain regions they might not be detected in a preparation from the whole forebrain.

In the next set of experiments the left and right IMHVs have been analysed instead of the whole forebrain. LPOs were not analysed in this thesis. Several modifications to the sample preparation were necessary. IMHVs are only about 5-10% of the whole brain, and the preparation of pure SPMs was not practical, because the yield of membranous material would have been very low. Therefore only synaptic plasma membrane enriched fraction, the crude synaptosomal fraction or P2 was prepared. This fraction is enriched in synaptic plasma membranes, however contamination due to mitochondria, glial cell membranes, myelin sheath fragments and microsomal membranes is also present.

Another modification concerns the extraction procedure. The chloroform:methanol extraction method was adapted to remove lipids from the membranous pellet as described by Finne (1982b). Glycans were released, labelled and separated exactly as in the previous sample set. The results are divided into the same categories i.e. the monosaccharide analysis is followed by the description of charged and neutral glycan profiles.

A note about the overlay chromatograms: traces corresponding to the "MeA trained" group always appear in grey, whereas "quiet control" chromatograms are always black. Only qualitative differences were examined, therefore the amount of samples injected (in picomols) was not determined (except when monosaccharides and sialic acids were quantitated). When quantitative

differences seem to be present (differences in peak areas) on the overlay chromatograms, they might be due to the different sample amounts injected.

9.1 Monosaccharide analysis of IMHV glycans

TFA release of monosaccharides

The monosaccharide composition of the IMHV P2 fractions was analysed in a similar way as previously described for the pure SPMs. There was no contamination from glucose, because a different membrane fractionation method was used, without the sucrose density gradient purification step. A standard mixture of monosaccharides was used for calibration and sample quantitation.

The delipidated glycoprotein fraction and not the 2-AB labelled glycan pool was hydrolysed; in this way a more accurate estimation was possible, because monosaccharides were released directly from the glycoproteins. Figure 9.1 and Figure 9.2 illustrate the overlay chromatograms of MeA trained and quiet chickens from the left and right IMHVs. Table 9.1 summarises the results of monosaccharide analysis expressed as nanomol/mg of dry protein as well as the relative ratios of individual monosaccharides relative to glucosamine.

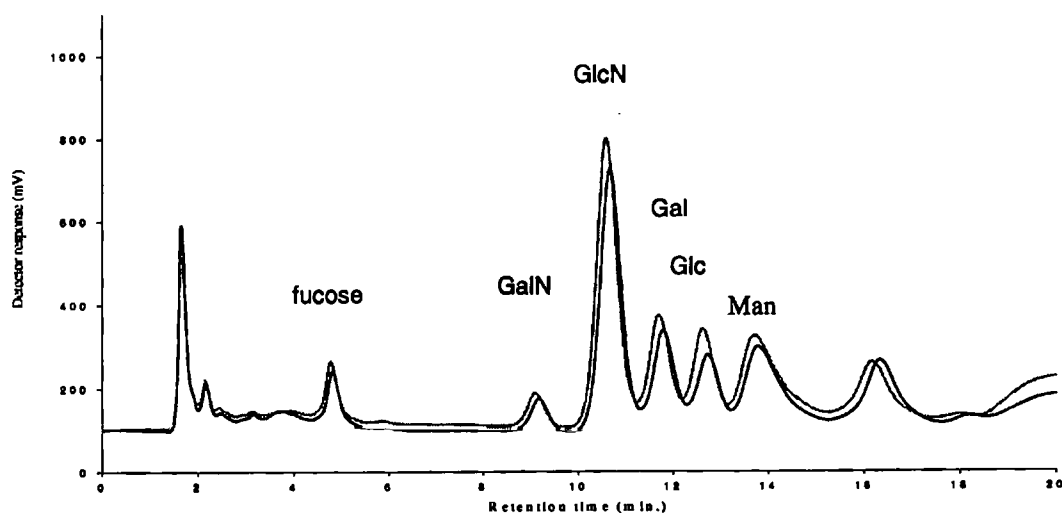


Figure 9.1. Left-hemisphere monosaccharide analysis from IMHV P2 fraction glycoproteins. Grey line: MeA trained, black line: quiet control.

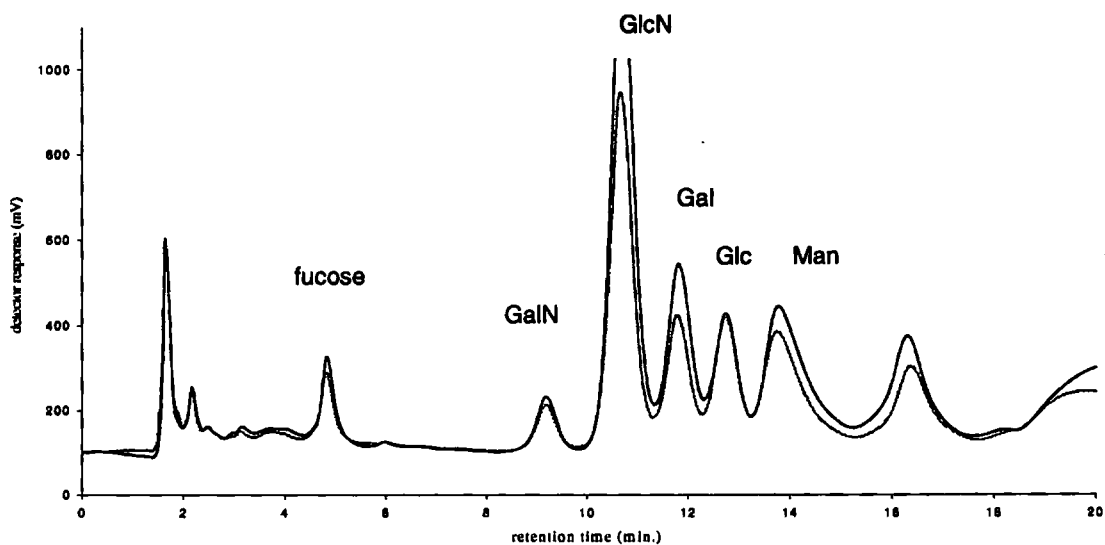


Figure 9.2. Right-hemisphere monosaccharides from the IMHV P2 glycoprotein fraction

Table 9.1. Monosaccharide analysis of IMHV P2 fraction glycoproteins

Left IMHVs				
Monosaccharide	MeA trained chicks		Quiet controls	
	nanomol/mg dry material	ratio to GlcN	nanomol/mg dry material	ratio to GlcN
Fucose	9.59	0.38	9.52	0.35
Galactosamine	2.55	0.10	2.99	0.11
Glucosamine	24.96	1.00	27.49	1.00
Galactose	12.64	0.51	14.01	0.51
Glucose	10.23	0.41	9.88	0.36
Mannose	18.23	0.73	18.94	0.69
Right IMHVs				
Monosaccharide	MeA trained chicks		Quiet controls ¹	
	nanomol/mg dry material	ratio to GlcN	nanomol/mg dry material	ratio to GlcN
Fucose	8.81	0.31	9.37	0.32
Galactosamine	3.37	0.12	3.11	0.11
Glucosamine	28.61	1.00	28.90	1.00
Galactose	14.00	0.49	15.52	0.54
Glucose	12.83	0.45	10.16	0.35
Mannose	19.56	0.68	19.32	0.67

Sialic acid analysis

Sialic acids were released by 0.1M HCl from the delipidated glycoproteins of the P2 fraction from the IMHVs, and analysed by HPAEC-PED under chromatographic conditions described in the methods chapter (p. 87). Standard mixture containing equal amounts of N-acetylneuraminic acid and N-glycolylneuraminic acid was separated before each sample set to identify and quantify the individual sialic acid species. Similarly to the

¹ Values are only approximate because of the GlcN peak area could be not exactly determined (Figure 9.2).

previous section, the results are presented as overlay chromatograms of MeA trained and quiet animals from the left vs. right IMHVs (Figure 9.3 and Figure 9.4 ; Table 9.2)

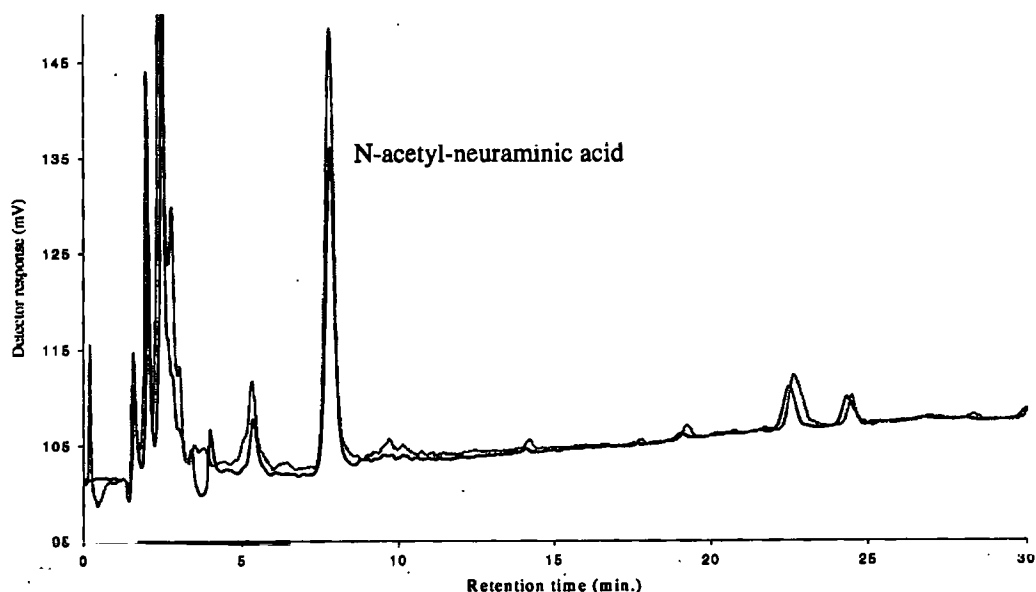


Figure 9.3. Left hemisphere sialic acid analysis from trained and quiet chicken IMHVs

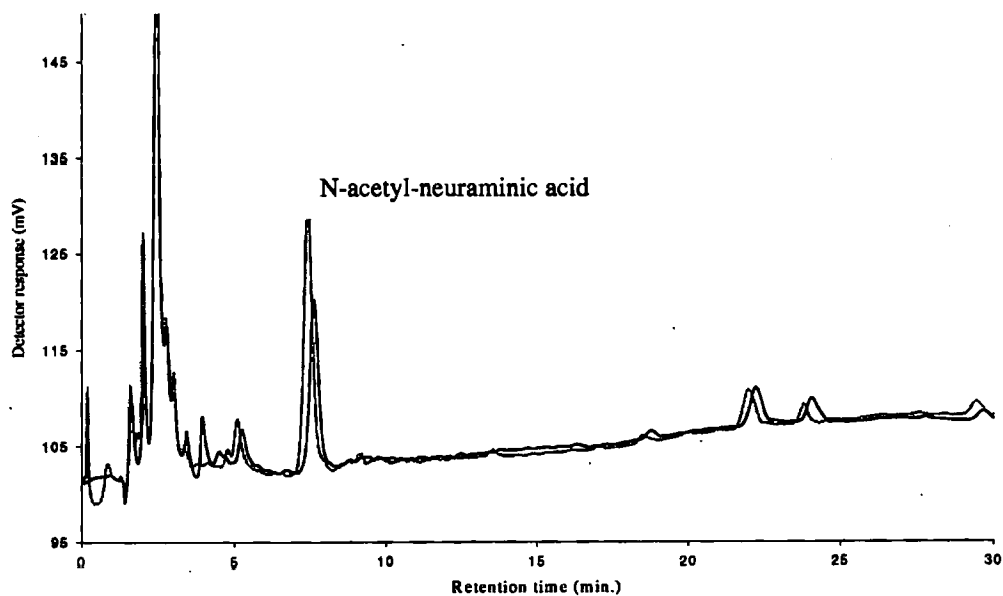


Figure 9.4. Right hemisphere sialic acid analysis from trained and quiet IMHV P2 glycoproteins

Peak areas were expressed as sialic acid content per milligram of dry material. The relative differences in the N-acetylneuraminic acid peak between the trained and quiet samples are misleading, as slightly different amounts of sialic acids were separated in the different

samples. However the exact amount of this starting material was known and taken into consideration during the calculations.

Table 9.2. Sialic acid content of the P2 fraction glycoproteins. Dry material represents the lipid-extracted P2 fraction.

	nanomol/mg dry material	µg NeuAc/mg dry material
MeA left IMHV	5.19	1.71
Quiet left IMHV	5.42	1.79
MeA right IMHV	3.71	1.23
Quiet right IMHV	3.91	1.29

Discussion

Monosaccharide analysis from the left and right IMHVs has identified the same six monosaccharide components which have been detected during the pure SPM sample analysis. The quantity of individual monosaccharides was in good agreement with the pure SPM sample set data (p.103), despite the fact, that glycoproteins (IMHV P2 fraction) as opposed to 2-AB labelled glycans (pure SPM) were used for the monosaccharide analysis.

The sialic acid content of IMHVs was similar to the pure SPMs. However, additional components were noted in the chromatographic profiles (peaks 22.7 min and 24.5 min), that were not present or at much lower levels in the pure SPM samples. The identity of those peaks is unknown (also compare Figure 3.2 p. 105 and Figure 9.3 p. 127).

A difference between the hemispheres was noted, as the sialic acid content in the right hemisphere was approximately 28% lower than in the left hemisphere (in both trained and quiet animals). Whether this is a reflection of hemispheric differences remains to be explored. However, a similar decrease in sialic acid content was noted in dark-hatched animals, a possible explanation of these findings will be presented later (p.140).

9.2 Charged glycan analysis of IMHV oligosaccharides

In the following experiments samples were analysed by weak anion-exchange chromatography, which separates glycans according to their overall charge. As neutral glycans elute as a single peak near the void volume of the column, this peak area can also be used to quantitate the neutral to charged glycan ratio in the original sample. A similar set of experiments (desialylation, dephosphorylation etc.) has been performed in order to characterise the nature of charged residues, as described in the previous, pure SPM sample set. However, sialic acid containing structures were analysed in more detail. This included the characterisation of α 2-3/8 vs. α 2-6 linked sialic acid distribution using linkage-specific sialidases, *Newcastle disease virus sialidase* (α 2-3/8) and *Arthrobacter ureafaciens* (α 2-3/6/8).

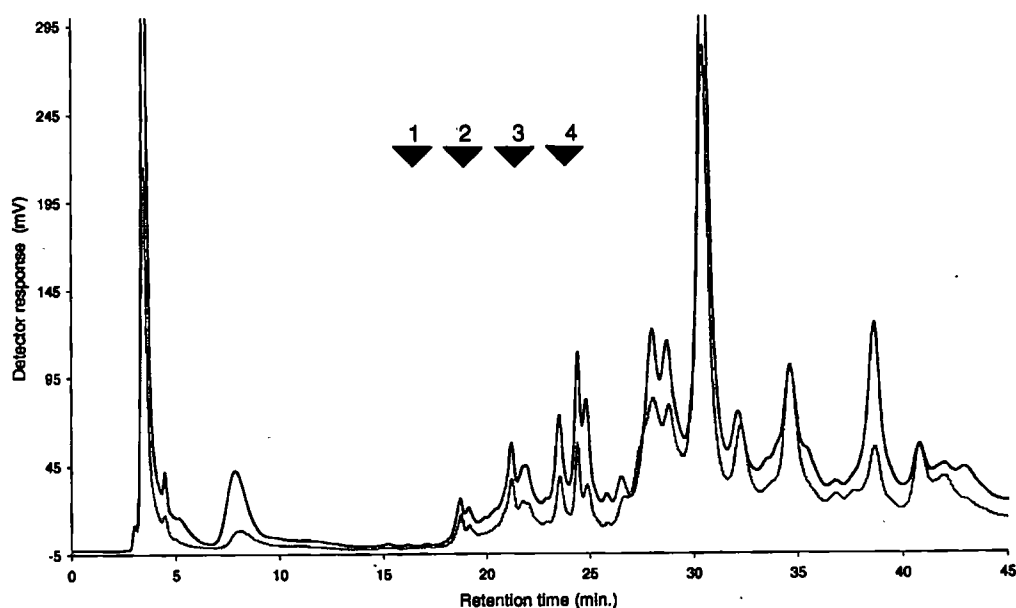


Figure 9.5. Overlay chromatogram of the total charged glycan pool from MeA trained and quiet animals (left IMHV). Arrows indicate the elution positions of mono-, di-, tri- and tetrasialylated fetuin standard.

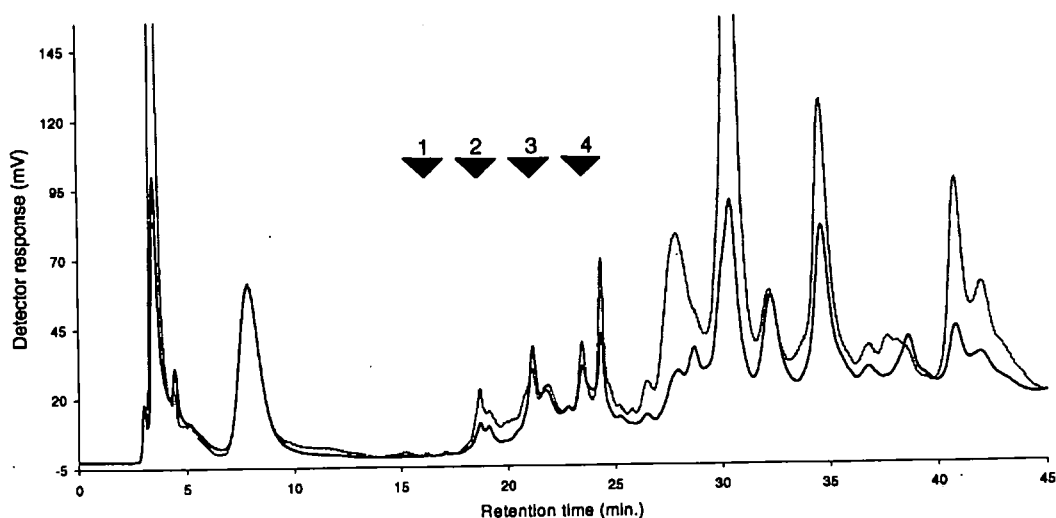


Figure 9.6. Overlay chromatogram of the charged glycans from MeA trained and quiet chickens (right IMHV).

The total charged glycan profile is more heterogeneous, when compared to the same glycan pool prepared from pure SPMs. The majority of peaks eluting after 25 minutes is sensitive to alkaline phosphatase digestion, indicating the presence of phosphate monoesters. However, a fraction of the highly charged material is sensitive to sialidase treatment.

Figure 9.5 and Figure 9.6 compare the total charged glycan profiles of MeA trained and quiet animals (left and right IMHVs, respectively). In the left IMHV, the profile is virtually identical, the same glycan species are present, with only a few showing possible changes in their relative abundance (peak 38.65). The right IMHV is however different. Although the same glycans are present, their relative ratios are more diverse than in the left hemisphere (Figure 9.6). Whether this is due to training on the passive avoidance task remains to be explored.

In general, structures co-eluting with di-, tri- and tetrasialylated fetuin standards could be identified. More negatively charged species were also present in significant amounts, eluting after 25 min. Most of these peaks disappear after alkaline phosphatase digestion,

indicating the presence of phosphate monoesters. Sulphate and phosphate were not quantitated individually for every sample during the IMHV sample set. Their contribution to the net charged glycan pool has been determined from the residual charged glycan pool present after sialidase digestions (Table 9.3 p.134).

In order to characterise in more detail the individual sialylated structures, the total glycan pool has been treated with two sialidases. *Newcastle disease virus* sialidase only cleaves $\alpha 2$ -3 and $\alpha 2$ -8 linked sialic acids, therefore peaks disappearing or shifting after digestion with this enzyme indicate the presence of $\alpha 2$ -3/8 linked sialic acid containing structures in the undigested pool. In a similar way, after digesting the already digested pool with $\alpha 2$ -3/6/8 specific enzyme (*Arthrobacter ureafaciens*) the shifting peaks are the indicatives of $\alpha 2$ -6 linked structures. The results are presented as overlay chromatograms for each individual sample analysed.

For all chromatograms:

- **dark line- untreated sample**
 - **light grey line – after Newcastle disease virus sialidase treatment ($\alpha 2$ -3/8 sialic acid)**
 - **medium dark- after Arthrobacter ureaf. sialidase treatment ($\alpha 2$ -3/6/8 linked sialic acid)**
- ↓ **$\alpha 2$ -3/8 linked sialic acid containing peaks**
- ▼ **$\alpha 2$ -6 sialylated structures.**

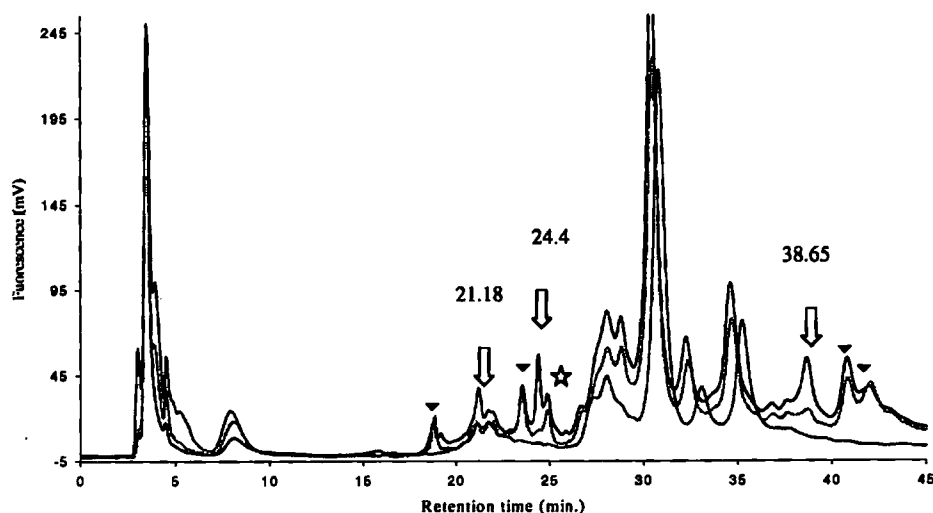


Figure 9.7. Sequential sialidase digestion of the glycan pool from the left MeA trained IMHVs. Thick arrows denote the $\alpha 2$ -3/8 sialylated structures, as they disappear or shift. Small triangles indicate the $\alpha 2$ -6 sialylated structures, as they are resistant to Newcastle disease v. sialidase digestion, but disappear after *Arthrobacter ureafaciens* sialidase digestion. Star denotes a peak eluting at 24.8 min., this peak is only present in the left hemisphere, and is $\alpha 2$ -6 sialylated.

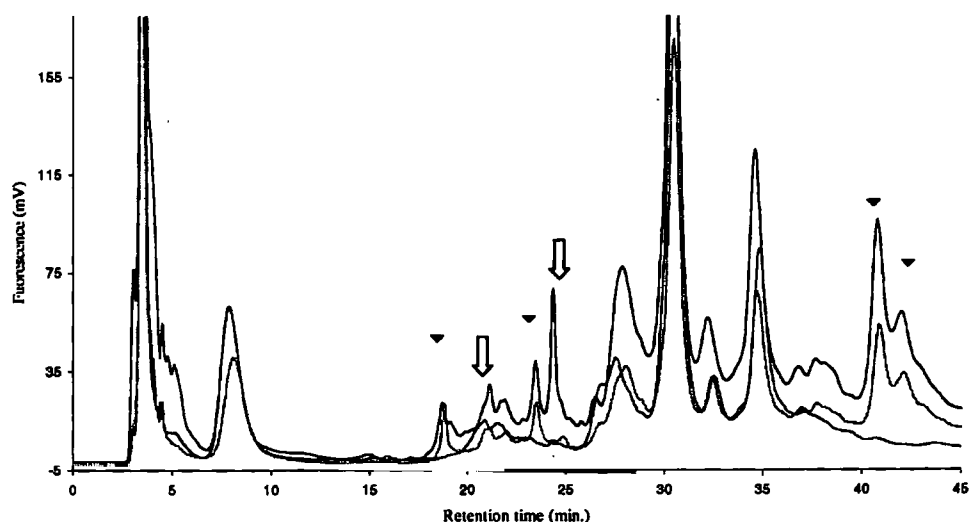


Figure 9.8. The $\alpha 2$ -3/8 and $\alpha 2$ -6 sialylated peaks are observed in the right hemispheres, except for the left-hemisphere specific 24.8 min. peak. Another peak which was sensitive to Newcastle disease virus digestion, but seems to be absent from the right trained hemisphere is the 38.65 min peak.

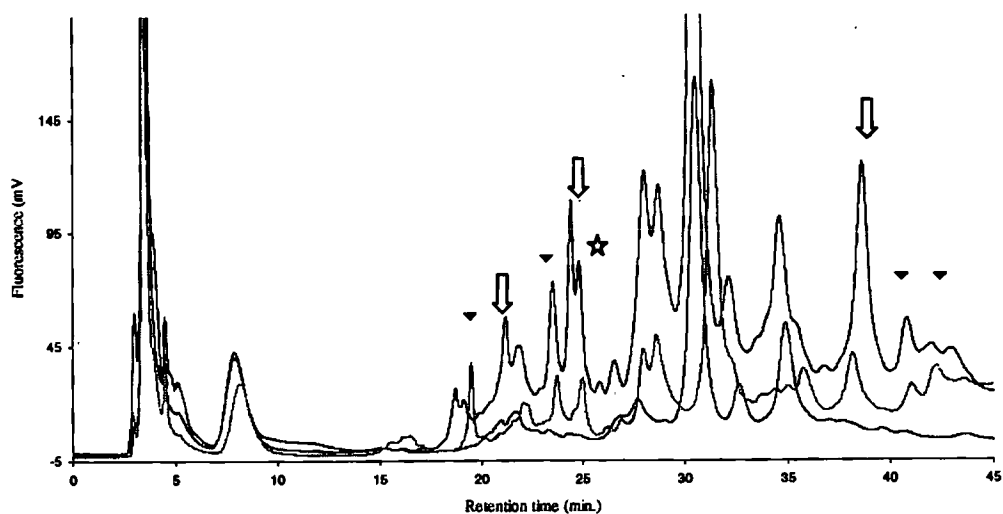


Figure 9.9. Left IMHV charged glycan analysis from quiet chickens.

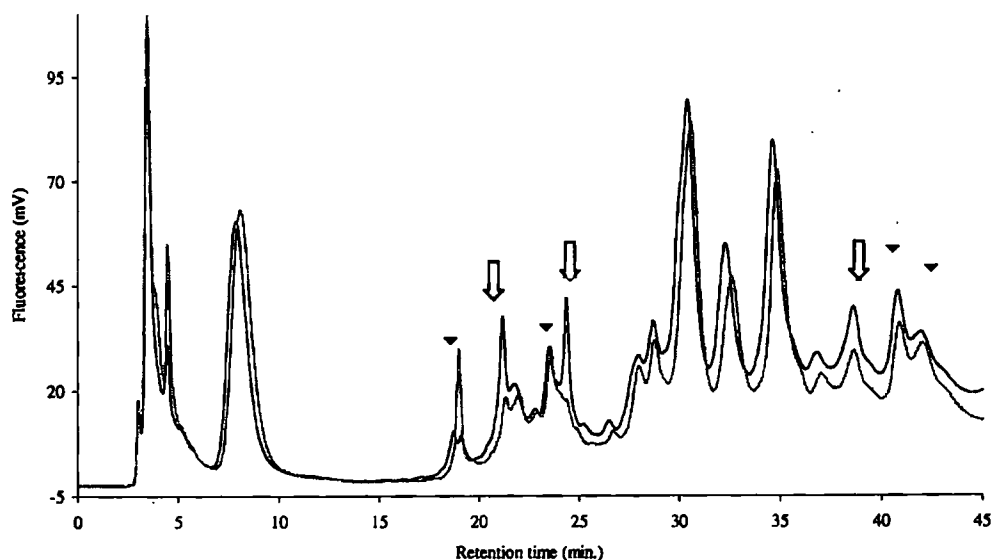


Figure 9.10. Right IMHV charged glycan analysis from quiet chickens. The chromatogram after the *Arthrobacter* sialidase digest is missing, only the total charged glycan pool and the Newcastle sialidase digests are shown.

In summary, the same $\alpha 2$ -3/8 and $\alpha 2$ -6 linked structures were found in all samples except for one peak eluting at 24.8 min, which was not present (or in very low amounts) in the right hemisphere. This peak is denoted by a star on the figures, and is identified as a tetrasialylated, $\alpha 2$ -6 linked sialic acid containing glycan.

Some sialidase sensitive peaks were also found in the highly charged region of the chromatogram (36-45 minute retention time). It is unfortunately not known where the polysialylated structures would elute from GlycoSepC under these conditions.

As the glycan profile was too complex to be compared at the level of individual peaks, only the gross contribution of α 2-3/8, α 2-6 sialic acid and (phosphate+sulphate) containing glycans to the net charged glycan pool was determined.

Table 9.3. Distribution of the charged and neutral glycan structures.

	Neutral %	α 2-3/8 linked sialic acid %	α 2-6 linked sialic acid containing structures %	Residual charged material (phosphate sulphate) %
MeA-left IMHV	10	8	11	71
Quiet left IMHV	12	10	15	63
MeA right IMHV	16	10	10	64
Quiet right IMHV	15	11	12	62

Discussion

The charged glycan profile from the IMHV P2 fractions had a more complex appearance than the same glycan fraction prepared from pure SPMs. It could be due to the differences in the starting material (whole brain pure SPMs vs. IMHV P2 fraction) as well as due to the differences during sample preparation (detergent extraction vs. delipidation).

The profiles were too complex to be compared between trained and quiet animals at the level of individual peaks, therefore only the relative ratios of neutral vs. charged glycans were determined. It is difficult to say, whether the differences on Figure 9.6 are the reflection of learning associated changes or due to standard experimental error. This methodology has not yet been used for similar purposes as described in this section; therefore further experiments would be required to determine the reproducibility of the

technique. Moreover, many of the interesting charged species are poorly characterised. Especially the supposedly phosphorylated glycans are present in large amounts, however it is not known, whether they are present on high-mannose structures or not. Some evidence suggests that phosphate is present on the oligomannosidic glycans (Finne 1990, Wing et al. 1992). Equally important would be to follow the subcellular distribution of these phosphorylated glycans, whether they are associated with the SPMs or whether they are contaminants from intracellular compartments.

From Table 9.3 it is evident that the majority (84-90%) of the glycan pool is charged. 19-24% of this total glycan pool is sialylated, 65-71% contains phosphate, sulphate and/or other charged residues. Czepita et al. (1994) found similar values in the mouse brain: 84% of the N-linked glycans were anionic, out of which 29% was sensitive to sialidase, 30% to hydrofluoric acid treatment (phosphate) and 26% to methanolysis (sulphate).

In summary, the significant proportion of other charged species in addition to sialic acid is surprising. Nothing is known about their identity, synthesis, distribution or possible function. These aspects would deserve further analysis, because if present at the synaptosomal plasma membranes in similar proportions as suggested above, they may significantly influence the biophysical properties of the neuronal cell membranes.

9.3 Neutral glycan profile of the IMHVs

The results from the pure SPM sample set suggested, that improved resolution is achieved by the combination of BioGel P4 and normal phase HPLCs (NP-HPLC). Therefore a preparative BioGel P4 was run to pool individual peaks in amounts sufficient for subsequent NP-HPLC analysis. These individual peaks were then analysed by normal phase HPLC and in some cases reversed phase chromatography.

As sufficient material was present in some of the most prominent peaks, they were sequenced as well (Chapter 11).

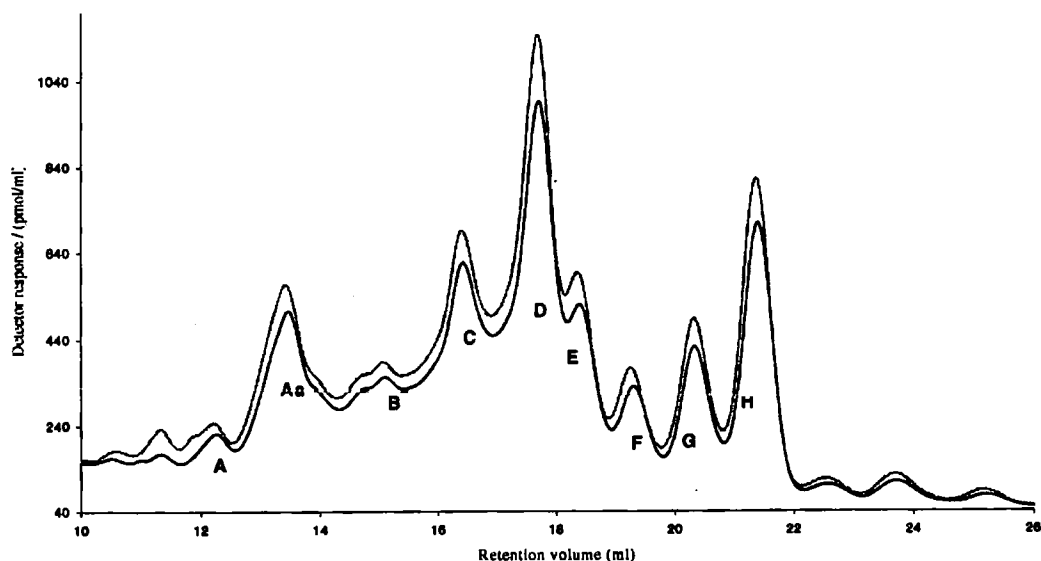
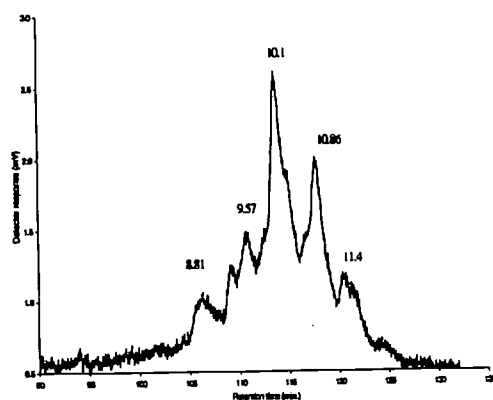


Figure 9.11. Preparative P4 chromatography of the neutral and neutralised glycans from the left IMHVs of trained (grey line) and quiet (black line) chickens.

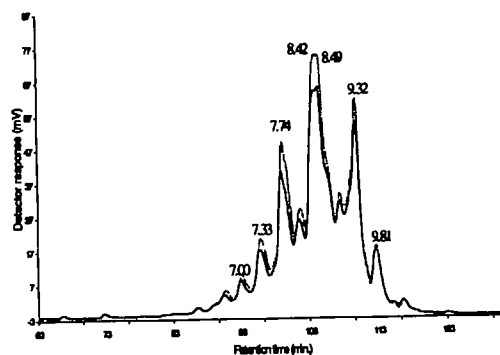
Table 9.5 Relative abundance and elution position of the P4 peaks from Figure 9.11

Peak	Ret. vol.	P4GU	Rel. area %
A	11-12.25	>20	5.15
Aa	13.46	19.21-16.52	11.54
B	15.1	14.42	6.82
C	16.43	12.08	13.47
D	17.71	10.49	17.15
E	18.39	9.51	5.86
F	19.3	8.51	4.07
G	20.33	7.71	5.54
H	21.39	6.86	9.41

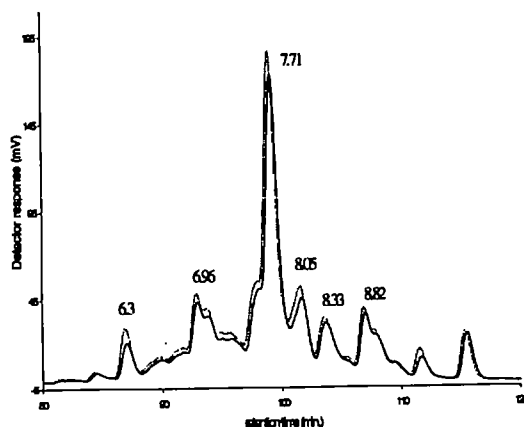
Individual P4 peaks were separated on GlycoSepN using the long-gradient for better resolution and easier sample pooling. Peak Aa has not been overlaid, but peaks B, C and D from the two sample groups were identical.



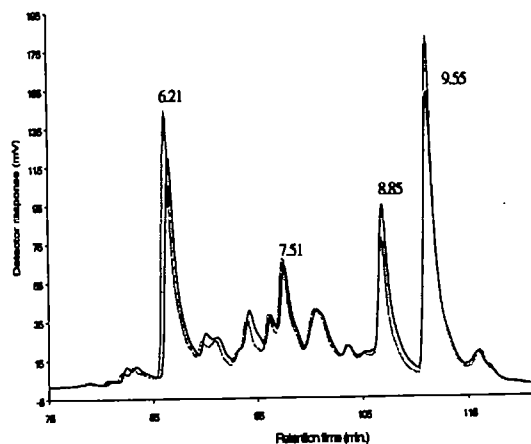
Peak Aa



Peak B



Peak C



Peak D

Figure 9.12. Overlay chromatogram of the major complex peaks from trained and quiet left IMHVs. Peak Aa was not overlaid. Grey line: MeA trained, black line: quiet chickens

Discussion

No difference was observed between the major neutral peaks analysed even when prepared from IMHVs and separated at high resolution by NP-HPLC. It is still possible that only individual glycoproteins are affected by training, and hence those changes diminish in the total IMHV glycan pool. Other alternative explanations are also plausible. As memory consolidation is a highly dynamic and temporal process, the time point chosen, 9 hours after training, may not be optimal. Or it is possible that only the turnover of the fucosylated glycans is affected, and not their total amount at the SPM. This possibility can not be analysed by the methodology used in the present thesis, as it would require (enzyme) kinetic measurements using (radio-)labelled sugar precursors.

To test the first hypothesis, that the IMHV glycan profile is still too complex compared to single glycoproteins, another experiment has been performed: the glycan profile of *individual* glycoproteins excised from PAGE gel bands have been compared between trained and quiet chicks.

Before that however two other experiments will be described, the first one is related to the issue of neural plasticity, but using a different experimental paradigm, that of dark-rearing. It is well documented, that the maturation of the visual system is partly input (light) dependent, but the role (if any) of particular glycan structures in this process has not yet been addressed. Therefore the glycan composition of the dark-hatched and normal, light-hatched chicken brain P2 fractions was compared.

The aim of the second experiment was to characterise in more detail some of the complex-type glycan structures described in the previous chapters.

10. Dark-hatched chicken brain glycan analysis

Introduction

The basic observation, that early visual experience can alter the structure and function of the brain has been verified in a wide variety of animal species.

Experimental data on the effects of dark rearing or exposure to extended time periods of light-deprivation are extensive. As an illustration, the following list covers some of the well-documented aspects of visual deprivation:

- lowered specificity for the orientation and direction of movement (Czepita et al. 1994)
- enlarged receptive fields (Fagiolini et al. 1997)
- altered firing rate (Benevento et al. 1992)
- impairment of spatially tuned auditory responses (Binns et al. 1995)
- altered cortical visual evoked potentials (Pizzorusso et al. 1997)
- altered proteoglycan expression in certain neuronal populations (Lander et al. 1997)
- decreased ganglioside glycosyltransferase activities (Bussolino et al. 1997)
- altered NMDA receptor mediated visual transmission (Catalano et al. 1997)
- decrease in muscimol binding (Gordon et al. 1997)
- lower cortex-thickness and vessel density (Argandona and Lafuente 1996)
- decreased MAP-2 positive dendrites (Reid and Daw 1995)
- decreased BDNF mRNA expression (Schoups et al. 1995)

Little is known about the glycosylation state of the neuronal cell membranes from dark-reared animals. The above-mentioned consequences of light-deprivation (e.g. neuronal communication, visual receptive field development) are processes, which presumably involve target selection and recognition in an activity-dependent fashion. It is one of the tenets of this thesis, that glycosylation may have an essential role in such processes. Therefore the dark-hatching paradigm was adopted to look at the possible effects of light-deprivation on the glycosylation profile of crude synaptosomal membrane fraction glycoproteins. Eggs were incubated and hatched under normal conditions (temperature, humidity and agitation) except for complete darkness. Age-matched animals were hatched

under the same conditions but with normal 12-hour day/night intervals. Food and water was provided ad libitum. Dark-hatched animals were decapitated in the complete absence of light. P2 fractions were prepared as described for the IMHV sample preparation.

Similar glycan analysis was performed as during the previous sample sets: sialic acid analysis after mild-acid release, preparation of 2-AB labelled glycan pool after hydrazinolysis, characterisation of the charge distribution in the total glycan pool, and comparison of the naturally neutral glycan pool.

10.1 Sialic acid analysis

Sialic acids were released by mild acid hydrolysis using 0.1M HCl and quantitated by HPAEC-PED as described in previous sections (Table 7.2 p. 87.). The results are summarised in Table 10.1.

Table 10.1. Sialic acid content of the whole brain (except cerebellum) P2 fraction glycoproteins.

	nanomol/mg dry material	µg NeuAc/mg dry material
Dark whole brain P2 fraction	3.68	1.22
Light-hatched whole brain P2 fraction	4.60	1.52
Left hemisphere from light-hatched chicks (IMHV)	5.42	1.79
Right hemisphere from light-hatched chicks (IMHV)	3.91	1.29

The results show a decrease in sialic acid content in the dark-hatched chicken P2 fraction from the whole brain. In the light of these results, it was also interesting to turn back to the previous sample set (left and right IMHVs). In the previous chapters, light-hatched animals were analysed, with special focus on training induced changes. However as a result of that analysis, it was possible to compare the hemispheric differences in sialic acid content of the light-hatched (quiet) control animals, those data are also presented in Table 10.1. A decrease in sialic acid content is evident in the right hemisphere, compared to the left hemisphere. A possible explanation of these results will be discussed below.

10.2 Charged glycan analysis

Glycans from dark-hatched and light-hatched controls were processed under identical conditions and separated first by weak ion exchange chromatography. Very similar profiles have been obtained, however, the ratio of neutral to charged glycans was different between the two samples (Figure 10.1). The large peak eluting at 7.8 minutes is the unreacted 2-AB dye used for fluorescent tagging of the glycans*. The areas of this 2-AB peak throughout the course of these studies have been carefully excluded during the peak area integrations. The relative areas of charged and neutral glycans are summarised in Table 10.2.

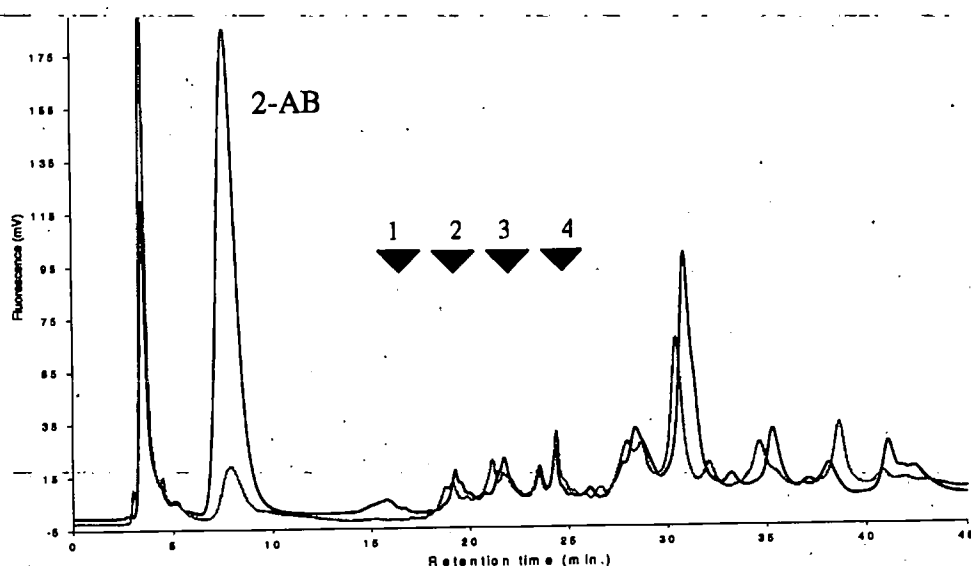


Figure 10.1. Charged glycan profile from dark-reared and normal chicken brain P2 glycoproteins. Lighter line: dark-hatched sample.

Table 10.2. Charge composition of the total glycan pool as determined from the peak areas after weak ion-exchange chromatography on GlycoSepC (from Figure 10.1).

	Dark-hatched whole brain P2	Quiet-whole brain P2
Neutral to charged ratio	0.49	0.20
Charged glycans	67%	83%
Neutral glycans	33%	17%

* The difference between the two samples is due to one omitted washing step during the cleaning up procedure after the labelling reaction, but this had no effect on the quality or quantity of the glycan profile

10.3 Comparison of the naturally neutral glycans from dark-hatched and control chickens

As can be seen from the previous analysis (Table 10.2), there was an increase in the neutral to charged glycan ratio in the dark-hatched chickens. This could be due to a decrease in sialylation/sulphation/phosphorylation of a *few* glycans or affecting the *majority* of the glycan pool. To examine these different possibilities, the neutral glycans were pooled during the GlycoSepC runs (peak eluting between 2.6-5 min. on Figure 10.1), and an aliquot of this material injected onto GlycoSepN.

Oligomannosidic structures with Man5-9 were the dominant glycans, as was the case in previous sample sets (Figure 8.13, p.116). Their relative ratios do not seem to be affected by dark hatching. The peaks present only in the dark-hatched samples around the 1.5-2 NPGU (Figure 10.2 b) elution position (7.4-11.8 min.) could be due to the presence of small O-linked structures.

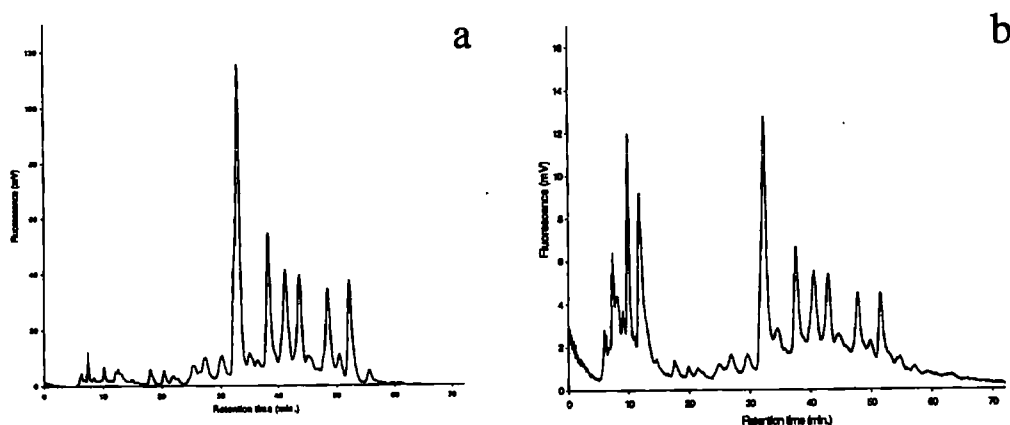


Figure 10.2. a.) neutral glycan fraction pooled during the weak ion-exchange chromatography from the light-hatched animals b.) from dark-hatched chickens. The small glycans present only in the dark-hatched sample could explain the altered neutral to charged ratio.

Discussion

Sialic acid analysis of the dark-hatched samples showed 20% decrease in total sialic acid content in the delipidated P2 fraction when compared to the light-hatched control P2 fraction (Table 10.1). Moreover, a similar decrease in the total charged glycan fraction

Dark-hatched

could be observed by comparing the peak areas after weak ion-exchange chromatography on GlycoSepC.

Judging from the naturally neutral glycan profiles, small, possibly O-linked structures seem to account for the altered neutral glycan ratio (Figure 10.2). O-linked glycans are typically sialylated at one or several terminal positions. It is possible, that these structures are undersialylated in the dark-hatched samples, because the sialic acid content is lower as well (Table 10.1). Unfortunately, at this stage no statistical significance can be associated with the results, because they have been performed only once. A careful repetition and additional analysis of the O-linked fraction would be required. A decrease in N-acetylmannosamine (sialic acid precursor) incorporation into the glycoprotein fraction of dark-hatched or dark-exposed chicks by (Caputto et al. 1982) is in agreement with the results presented above.

It is also interesting to look in more detail at the sialic acid content of the left and right hemisphere from normal, light-hatched control chickens. In the right hemisphere, the sialic acid concentration has always been lower than in the corresponding left hemisphere. The left eye receives less light stimulation during hatching because of the position of the chick in the egg (Figure 10.3.) It would have been interesting to follow the *hemispheric* differences in the dark hatched animals as well. On the basis of these results one would not expect to find a decrease in sialic acid content in the left hemisphere, as there would have been no difference between the amount of light stimulation during hatching. However, there are hemispheric differences in dark-hatched chicks in the level of MK801 binding (Johnston et al. 1995). Moreover, there may be sex-related differences in the distribution of charged glycoconjugates between the hemispheres. Female rat brains have higher ganglioside content in their right hemispheres, whereas male rats show no hemispheric difference (Palestini et al. 1997).

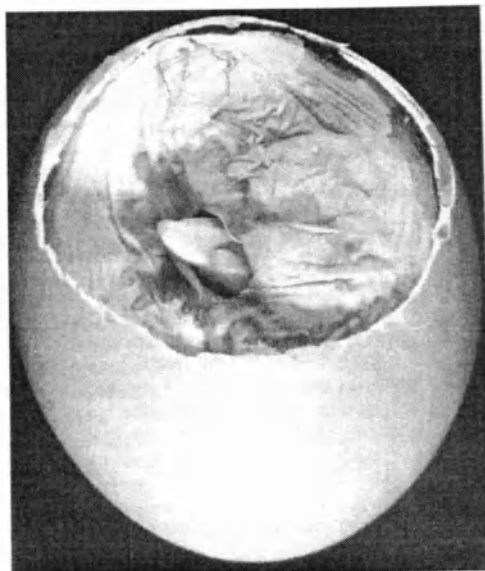


Figure 10.3. The position of the chick embryo in the egg during development (E20). Note the position of the left and right eye (from Rogers (1995)).

A similar trend in the “dark-light” vs. “right-left hemisphere” sialic acid content would suggest that the total negative charge carried by the membrane associated glycoproteins can be influenced by the amount of light stimulation. In this regard it is interesting that ganglioside glycosyltransferase activity in the retinal ganglion cells is stimulated by light exposure of dark-hatched chickens (Bussolino et al. 1997).

One can also speculate about the possible interactions of corticosteroids with the glycosylation machinery/profile in the nervous system. Corticosterone injection, as well as light-stimulation during E19 and E20 of the chicken development can compensate for the amnesic effects of dark-hatching on memory formation for the passive avoidance task (Sui and Rose 1997, Sui et al. 1997). Corticosteroids have complex, but well documented effects on sialyltransferase activities in the brain (Coughlan 1996, Maguire et al. 1997). It would be therefore worthwhile to explore the possible effects of corticosteroids on the individual sialyltransferase activities in dark-hatched and normal chicken brains.

11. Glycan sequencing

After the detailed analysis and comparison of the charged and neutral glycan fractions from the two experimental groups the IMHV P2 fractions were pooled and converted to neutral glycans. This glycan pool was fractionated by gel-permeation chromatography (Figure 11.1a.). Five major P4 peaks (Aa, A, B, C and D) have been pooled in sufficient amounts to allow sequence analysis. However, only the major components of peaks C and D were characterised in detail.

Sequential enzymatic sequencing can only be used for individual oligosaccharides, as it does not allow the simultaneous sequencing of multiple glycan structures. Therefore P4 peaks were further fractionated into individual components using normal-phase HPLC (Figure 11.1). The homogeneity of the NP-HPLC fractions was tested by reversed-phase and if necessary, another preparative step was included in this mode (data not shown). Two different methods were applied, sequential enzymatic sequencing for peak D and RAAM for peak C (p.83).

11.1 Peak D

Peak D pooled during a preparative gel-permeation chromatography on P4 was separated in more detail on GlycoSepN. Individual components (peaks D1, D2, D3, D4, D5, D6, D7 and D8) have been pooled and desalted by repeated rotary evaporation from pure water. From their relative abundance and NPGU values, peaks D7 (8.85 NPGU) and D8 (9.56 NPGU) were suspected to be Man₈ and Man₉ structures, respectively. Therefore an aliquot (~15 pmol) of the pooled peaks was digested with jack bean α -mannosidase, with specificity for $\alpha(1,2/3/6)$ Man (see digestion conditions on p.97). The digests were desalted, deproteinated and analysed by NP-HPLC on GlycoSepN. Both D7 and D8 shifted to the same 2.3 NPGU structures, which is almost certainly Man β 1-4GlcNAc β 1-4GlcNAc. Empirically determined NPGU values of individual monosaccharides have been used to

interpret the results of individual digestions. These values were adapted from Chen et al. (1998), and are listed in table Table 11.1.

From the differences in the NPGU values of D7 and D8 before and after digestion with the α -mannosidase it is suggested, that D7 is an isomer of Man8 and D8 of Man9 structure.

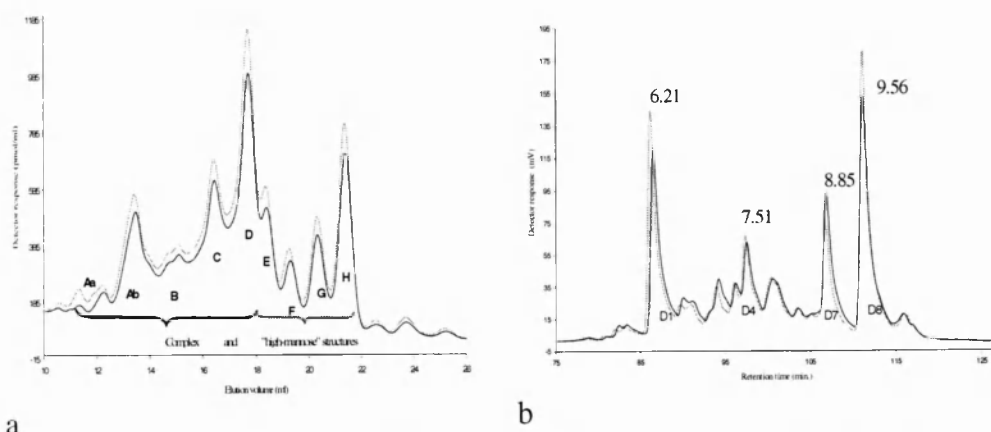


Figure 11.1 Single peak D (a) separated into individual components by normal-phase HPLC (b)

Table 11.1 Incremental values of individual monosaccharides on normal-phase HPLC

Monosaccharide	NPGU incremental value
Gal	0.8-1.1
Man	0.75-1.0
GlcNAc	0.55-0.75
Bisecting GlcNAc	0.08-0.39
Core $\alpha(1,6)$ Fuc	0.4-0.45
Outer-arm $\alpha(1-3)$ Fuc	0.7-0.9

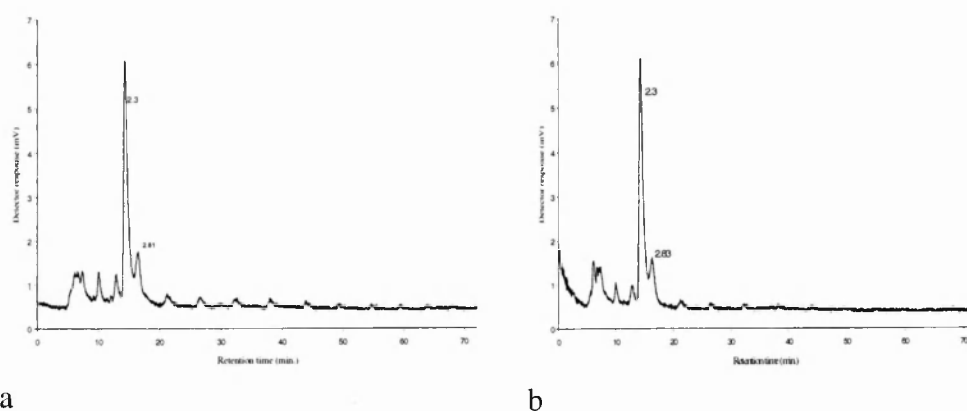


Figure 11.2 Jack bean α -mannosidase digestion of peaks D7 (a) and D8 (b). the enzyme converted both peaks to the same small structure eluting at 2.3 NPGU.

Peak D1 has also been digested with jack bean α -mannosidase, however no shift in its elution position was observed indicating that it is not an oligomannosidic structure. Next, D1 was digested with 0.01 U/ml *S.pneumoniae* β -hexosaminidase. At this enzyme concentration the enzyme will cleave terminal GlcNAc residues, except when a) the $\beta(1-2)$ GlcNAc is attached to a mannose which is also substituted at the C6 position; b) $\beta(1-2)$ GlcNAc is not cleaved on the $\text{Man}\alpha(1-6)$ arm in the presence of a bisecting GlcNAc. At higher enzyme concentrations (0.1 U/ml) this fine specificity is lost, and all terminal GlcNAc residues are removed. This property of the enzyme is especially useful for identifying structures with bisecting GlcNAc.

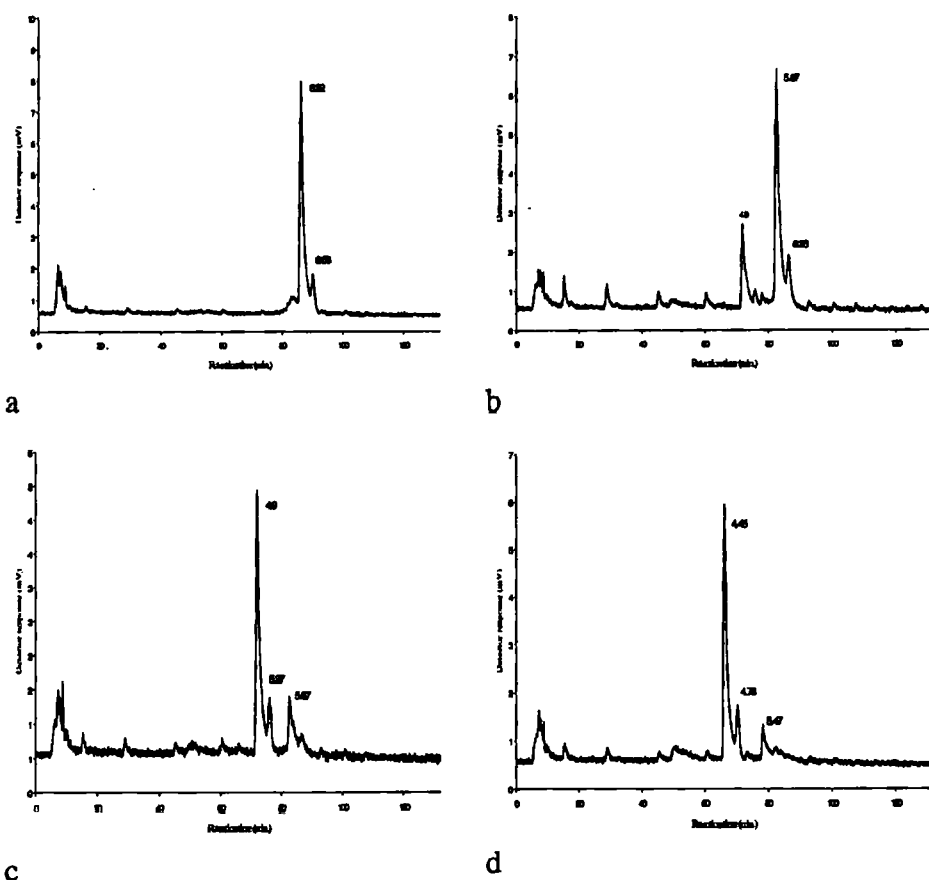


Figure 11.3. Sequential sequencing of D1: a) untreated sample; b) 0.01 U/ml *S.pneum.* hexosaminidase; c) 0.1 U/ml *S.pneum.* hexosaminidase; d) 0.1 U/ml *S.pneum.* hexosaminidase and bovine kidney fucosidase.

The results of individual digestions are summarised on Figure 11.3. *S. pneumoniae* hexosaminidase at 0.01 U/ml concentrations removed a single glucosamine residue (shift of 0.35 NPGUs). Digestion with the same enzyme at higher enzyme concentrations

(0.1U/ml) however resulted in a shift of 1.32 NPGUs, which can be explained by the removal of 3 GlcNAc residues.

Bovine kidney fucosidase removes terminal and core fucoses in α 1-2/3/4/6 linkages. Incubation with this enzyme following hexosaminidase treatment resulted in a shift corresponding to 1 core fucose residue (0.45 NPGU). The final structure eluted at 4.45 NPGU, which corresponds to the trimannosyl core structure. On the basis of these data it can be suggested that D1 is a bisecting biantennary, core fucosylated structure (Figure 11.4).

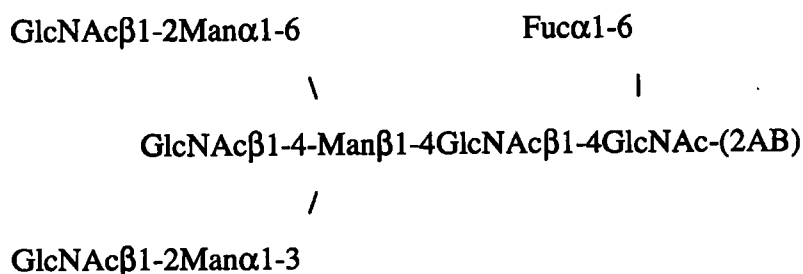


Figure 11.4. Proposed structure of peak D1.

11.2 Peak C

The major component of peak C has also been sequenced, however with a different method, using the RAAM 2000™ GlycoSequencer (Oxford GlycoSciences).

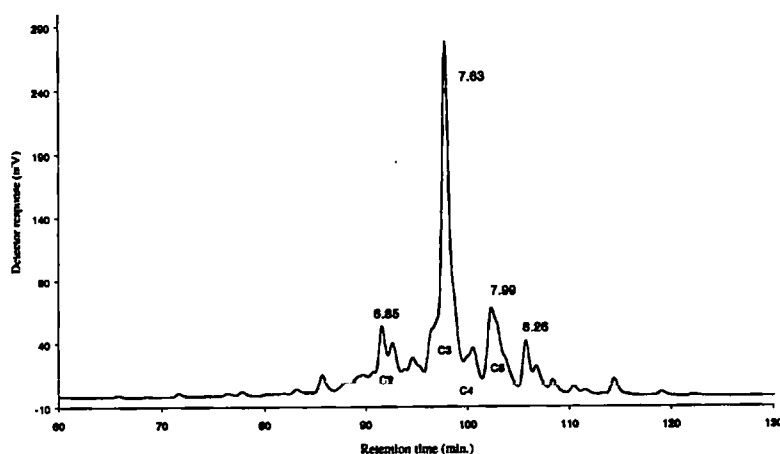


Figure 11.5. Peak C from P4 chromatography separated by normal-phase HPLC on GlycoSepN. The major component (C3) eluting at 7.63 NPGUs was pooled and sequenced by RAAM.

100 picomols of the C3 peak (7.63 NPGU) was used for sequencing according to the manufacturer's instructions using the sequencing kit for neutral N-linked glycans.

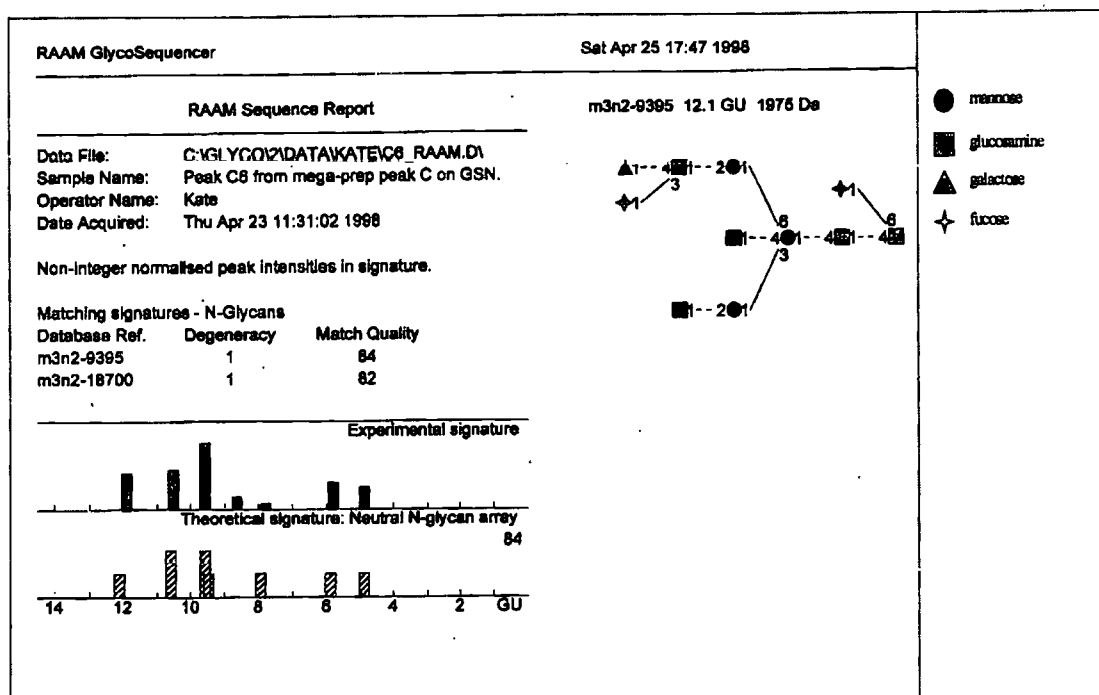


Figure 11.6. The RAAM sequence report for peak C.

After incubation with the enzyme arrays and desalting, the combined digests were separated on P4 using the GlycoSequencer. The chromatogram was converted to an “experimental signature” by the Sequencer’s built-in software, and compared to all the theoretically possible signatures. The best matches were then listed and assigned a value of match quality using the Kolmogorov-Smirnov statistic. As can be seen on Figure 11.6, the best match is 84%, which is within the “good” confidence limit. Therefore the structure illustrated on Figure 11.6 can be considered as the sequence of peak C3.

Discussion

The sequence of the D1 glycan is interesting because the same structure was found to be the predominant *neutral* glycan in mouse brain cerebellum, cerebrum and brain stem (Shimizu et al. 1993). This structure seems to be specific to the nervous system. Shimizu et al. (1993) were looking for possible tissue specific glycan structures by comparing the neutral glycans from several mouse tissues (liver, heart, spleen, kidney, thymus, cerebrum,

cerebellum, brain stem). They found D1 to be specifically enriched in the cerebrum, cerebellum and brain stem.

A year later, Hoffmann et al. (1994) have undertaken the detailed glycan analysis of a glycoprotein synthesised in the human brain and secreted to the cerebrospinal fluid. Surprisingly, the predominant glycan had a structure corresponding to D1. The same authors later characterised another glycoprotein from the human cerebrospinal fluid, asialo transferrin (Hoffmann et al. 1995), and D1 was found to be one of the predominant structures again.

The first paper providing a systematic and comprehensive analysis of the neutral glycan structures present in the adult rat brain has been published only very recently by Chen et al. (1998). Again, structure D1 was the second most abundant N-linked complex-type neutral glycan.

These findings, together with the data presented above about the chicken brain glycan structures suggest a specific role for this particular structure in the nervous system.

It is difficult to speculate about its potential function. There are suggestions, that the presence of bisecting GlcNAc may be important during cell sorting or even intracellular trafficking (Sultan et al. 1997), in the modulation of Trk tyrosine phosphorylation (Ihara et al. 1997), or modulating the glycosylation pathway (p. 57.).

The sequence obtained for peak C3 is in good agreement with the results from the rat brain (Chen et al. 1998), where the same sugar is the third most abundant complex type glycan component. Unfortunately the mouse brain was not studied by Shimizu et al. (1993) in more detail, therefore it is not known, whether this glycan is present or not. An interesting feature of peak C is the presence of the LewisX (CD15, SSEA-1, FAL) determinant. As monoclonal antibodies were available against this specific sugar epitope, the identity of SPM glycoproteins carrying this epitope was investigated by Western blotting.

11.3 Western blotting with anti-CD15 monoclonal antibody

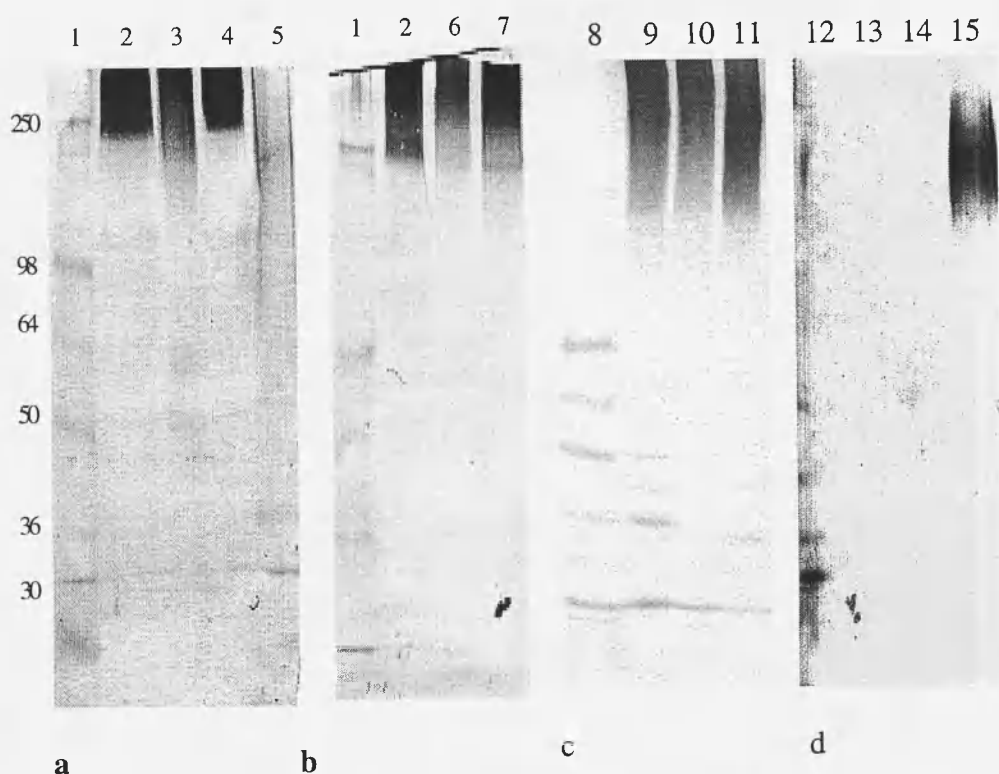


Figure 11.7 Western blots with CD15 monoclonal antibodies recognising the LewisX sugar epitope (a-c) and with anti-sialyl-LewisX antibody (d).

Blot a) 1: Mw standards; 2: whole brain P2 fraction; 3: digested with *Arthrobacter ureafaciens* sialidase; 4: blank digested control sample; 5: control HL60 cell hydrolysate.

Blot b) 1: Mw standards; 2: untreated whole brain homogenate; 6: endo- β -galactosidase (from *Sterptomyces*) digested sample; 7: blank digested control sample

Blot c) 8: Mw standards; 9: whole brain P2 fraction; 10: blank; 11: chondroitinase ABC treated whole brain P2 fraction

Blot d) 12: Mw standards; 13: left IMHV P2 fraction; 14 whole brain homogenate; 15: HL60 cell lysate

Interestingly, the antibody recognized only a limited range of high molecular weight proteins, which are -judging from their migration position and appearance- proteoglycans or glycoproteins with large polymeric (polylactosamine?) glycan structures.

In order to characterise the CD15 positive glycoproteins/proteoglycans, the chicken brain glycoproteins were treated with several enzymes before Western blotting with anti-CD15 mAb.

The first enzyme tested was the *Arthrobacter ureafaciens* sialidase (Figure 11.7a). Removal of the sialic acid residues resulted in a more disperse appearance of the CD15 positive glycoproteins. This result indicates that the glycan structures associated with the

CD15 positive glycoproteins/proteoglycans are sialylated. HL60 cell line was used as positive control for its known expression of LewisX (Jacob et al. 1995).

Endo- β -galactosidase was the next enzyme to test for the presence of polylactosamine like structures, and whether the LewisX antigen is associated with these glycans. As can be seen on Figure 11.7b line 6, there was indeed some reduction in the intensity of CD15 staining, indicating, that at least a fraction of LewisX is presented on polylactosamine like structures. Because only one enzyme concentration was tested, the results may reflect partial digestion. In two independent studies, the LewisX epitope could be completely removed by endo- β -galactosidase digestion from a similar >220 kDa proteoglycan phosphacan (Childs et al. 1983, Allendoerfer et al. 1995).

From their appearance and especially high molecular weight, it was suspected that CD15 is present on proteoglycan type molecules. As chondroitin sulphate (CS) is the most abundant proteoglycan species in the brain, an aliquot of the whole brain P2 fraction was treated with chondroitinase ABC. No loss in the intensity of CD15 staining was observed, however, the migration of the protein has slightly increased. This would suggest that the Lewis X epitope is not present on the chondroitin sulphate side chains, but the CD15+ protein is a proteoglycan, containing CS GAGs. The same shift in migration position upon chondroitinase ABC treatment was observed in the rat brain with the FORCE-1 antibody, recognising the LewisX epitope (Allendoerfer et al. 1995).

Closely related to Lewis X is its sialylated homologue, S-LewisX. In many tissues types LewisX and sialyl-LewisX are often co-expressed, except in the CNS, where both antigens seem to be differentially expressed (Gocht et al. 1996). As illustrated on Figure 11.7d, no immunoreactivity could be detected in the IMHV P2 fractions, indicating that sLeX is probably not expressed at significant levels in the day-old chicken brain SPMs. HL-60 cells were used as positive control (Figure 11.7, line 15).

The region of the Coomassie blue stained gels corresponding to the CD15 immunoreactivity on Western blots contains only a limited number of proteins (e.g. Figure

12.1, p.159). Therefore, to get some information about the possible identity of the CD15+ species, the uppermost 4 bands were protein sequenced directly from PAGE gels. The following information has been provided by Dr Malcolm Ward's protein sequencing group (GlaxoWellcome, Stevenage) about the possible identity of these bands:

Homologue of human gene product KIAA0325 - Genebank accession number AB002323
Claustrin - Genebank accession number X67778
Sodium/Potassium-transporting ATPase alpha chain
ATN1 P09572; ATN2 P24797; ATN3 P24798 Genebank accession number

Prior to protein sequencing, on the basis of literature survey it was suspected that the CD15 positive band might be the brain specific chondroitin sulphate proteoglycan phosphacan. After PAGE separation, phosphacan appears as a large >220 kDa, disperse band expressing the LewisX epitope. More importantly, a similar behaviour of that protein is observed when treated with the same enzymes, as described on Figure 11.7, (Allendoerfer et al. 1995). Therefore it cannot be ruled out that the CD15 positive chicken glycoprotein is phosphacan, despite the fact that sequencing the corresponding PAGE bands failed to confirm its presence. Or equally true, there might be other, as yet uncharacterised CS proteoglycans in the brain, expressing the CD15 epitope.

What is known about the sequenced proteins? The first candidate, *AB002323* was isolated from a human male brain cDNA library. Genebank search for this structure identified dynein heavy chain 1a isotype as a possible homologue, and nothing is known about the protein product and its glycosylation profile.

Claustrin has been identified as a neural tissue specific keratan sulphate proteoglycan primarily produced by astrocytes and excreted to the extracellular medium (Cole and McCabe 1991). It has neurite outgrowth inhibitory properties and is not known, whether it carries the LewisX epitope.

The *Na⁺/K⁺ ATPase* alpha subunit (in contrast to the beta subunit) is not glycosylated in chicken sensory neurons (Tamkun and Fambrough 1986).

The last three sequences (*P0572*, *P24797* and *P24798*) are homologous to the chick sodium/potassium transporting ATPase alpha1, 2 and 3 mRNA chains.

These results do not provide helpful information. Immunoaffinity isolated glycoproteins (on CD15 affinity column, or by immunoprecipitation) would have been a more appropriate starting material for sequence analysis. Other experiments are required to characterise the identity of the chicken CD15 positive chondroitin sulphate proteoglycan.

The glycan epitope recognized by the monoclonal antibody used in the Western blot experiments described above is the α -1,3fucosyl lactosamine trisaccharide. (LewisX). This glycan was found on the C3 glycan component on the 1-6 arm of a bisecting, biantennary, core fucosylated structure (Figure 11.6). Although C3 is one of the most abundant complex type structures found in the chicken brain, it does not necessarily indicate that the monoclonal antibody recognises the C3 glycan structure on Western blots. LewisX can be present on a very large number of different N-linked glycans (Chen et al. 1998), moreover as has been discussed previously (see p.60), different antibodies may have different affinities towards the same antigen, depending on the actual microenvironment of the glycan. Several monoclonal antibodies against CD15 should have been tested in parallel, however this was not possible for several reasons. Again, purification of the CD15 positive proteoglycans and subsequent glycan analysis would be required to characterise in more detail the exact structure of the glycan antigen.

Nevertheless, the results presented certainly confirm the presence of the LewisX epitope on an N-linked glycan structure. Many other structures are expected to carry the LewisX epitope (Chen et al. 1998), their identification in the chick requires further analysis.

Regarding the self-adhesive properties of the LewisX epitope in the presence of M^{2+} cations, it cannot be *a priori* ruled out that LewisX positive structures are present as aggregates. Other experiments investigating the effect of EDTA and/or the inclusion of

chaotropic agents, like urea, in the sample buffer or in the polyacrylamide gel prior to electrophoresis would be useful to perform.

No CD15 reactive protein bands corresponding to L1, N-CAM, MAG or J1 were observed. These cell adhesion molecules have been suspected to carry an N-linked glycan component with the LewisX determinant (Wing 1992).

After these experiments a new method designed for the glycan analysis of PAGE protein bands directly from the protein gels was tested for possible future applications.

12. Analysis of N-linked oligosaccharides directly from protein gels

12.1 Introduction

Glycan analysis did not detect any difference between the neutral and neutralised glycan pool. One reason for that could be the unsuitability of this method at the level of total glycan pool, even when it was derived from isolated brain regions like IMHV. Therefore the next step would have been the glycan analysis at the level of *individual glycoproteins*. Such a preparation would however require immunoprecipitation or affinity chromatography using monoclonal antibodies against the individual glycoproteins. Such antibodies were not available at the time of the studies.

Preparative PAGE electrophoresis has also been considered, however the resolution power of this one-dimensional method would not have been appropriate for a glycoprotein mixture as diverse as the synaptic plasma membrane fraction where the fucosylated glycoproteins are not necessarily the major Coomassie stained proteins.

Bullock et al. (1992) have separated the radioactive fucose labelled glycoproteins from the LPO synaptic plasma membrane fraction by PAGE electrophoresis, and measured the radioactivity in individual Coomassie blue stained gel slices. In this way, they were able to identify those Mw bands, which showed altered fucose incorporation after training. A similar method for the glycan analysis of these protein bands has also been considered. Fortunately, during the analysis of the IMHV samples a novel method suitable for the glycan analysis of individual PAGE gel bands has been published. The method uses a very similar glycoanalytical approach (2-AB labelling and subsequent HPLC separation and/or MALDI-MS) as described in the previous chapters.

In order to test the suitability of this method for possible application to a PAGE separated chicken SPM glycoprotein analysis, well-characterised glycoproteins (fetuin and RNaseB)

were first analysed. Because the method gave excellent results for these two glycoproteins, two additional experiments using chicken IMHV SPM glycoproteins implicated in the memory processes were performed.

In the first, the glycosylation profile of three learning associated glycoproteins (33 kDa, 45 kDa and 180 kDa as detected by Bullock et al. (1992) was compared between trained and quiet animals from left IMHV P2 fraction.

In the second experiment, the major fucosylated glycoproteins, as identified by a panel of fucose specific lectins were characterised in detail.

12.2 Individual glycoprotein analysis from PAGE gel bands-a novel method

The practical details of the in-gel deglycosylation procedure are summarised next. The method was adapted from Kuster et.al (1997).

Solutions

20 mM NaHCO₃ (Fisher) (M_w=84.01)

45 mM dithiothreitol DTT (Sigma) M_w=154.2 (0.168g/ 10 ml)

100 mM iodoacetamide (Sigma) M_w=185 (0.185g/10 ml)

acetonitrile (BDH)

Gel running buffer (BDH)

25 mM TRIS

190 mM glycine

0.5% SDS

Polyacrylamide stock solutions

100 ml of 40% w/vol acrylamide (40 g/ 100 ml)

20 ml of 2% w/vol bis (0.4 g/ 20 ml)

Protein stain

0.1% Coomassie blue (Sigma)

Destain

30% methanol, 7.5% acetic acid

Sample Preparation

100 μ g of fetuin and RNaseB (Sigma) was separated by PAGE.

Sample preparation of the chicken left IMHV P2 fractions

5 mg of delipidated left IMHV P2 fractions was dissolved in 100 μ l 0.5M Tris pH 7.4 and 200 μ l 20% SDS by rotating overnight at 4°C.

Lectin-positive samples

In order to pool sufficient amount of individual gel bands, three identical gel lines were run for the MeA trained and quiet samples, which were then pooled and processed together (Figure 12.1a). Eighty μ g protein was separated in each line.

Learning-related glycoprotein fractions

A modification of the above strategy was to use wide-comb gels (600 μ g/gel), and therefore single gel stripes were cut out for in-gel digestion and glycan analysis (Figure 12.1b).

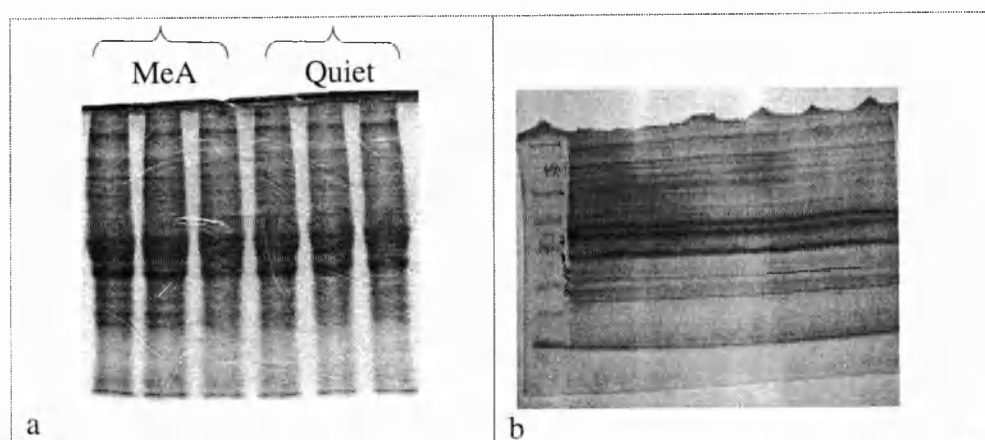


Figure 12.1. The process of sample preparation for a) lectin positive glycoproteins: individual PAGE bands were cut from 3 gel lines and pooled for further analysis. b) learning-associated glycoprotein bands (33, 45 and 180 kDa) were prepared using wide-comb gels.

Gel Electrophoresis

In order to improve the efficiency of the in-gel PNGaseF digestion, the polyacrylamide gel had lower *bis*-concentration (0.1%) and hence lower density without affecting its separation properties (Kuster et.al 1997).

12.5% resolving gels were made in BioRad gel-casters, with 5% acrylamide 0.13% *bis*-acrylamide stacking gel composition (Table 12.1).

Table 12.1 Composition of the PAGE gels used during the in-gel –deglycosylation process

	12.5% acryl/ 0.1% bis	17.5% acryl / 0.07% bis	5% acryl/0.13% bis (stacking)
40% acryl	12.5	17.4	1.25
2% bis	2	1.4	0.65
1.5M Tris-HCl pH 8.8	10	10	2.5
H ₂ O	15	10	4.86
TEMED	75 µl	75µl	24µl
10% APS	132µl	132µl	50µl

Separation was at 150 V constant voltage in 25 mM TRIS/190 mM glycine/0.5% SDS.

Protein Staining

After separation the proteins were visualised by overnight Coomassie staining. Background staining was removed by repeated rinsing in 30% methanol and 7.5% acetic acid. Individual protein bands were excised by a sterile scalpel or razor. Special care was taken to avoid any source of glycoprotein or glycan contamination during sample handling (e.g. by using powder-free gloves). Individual protein bands were further destained in 70% methanol, 7.5% acetic acid by several washings in an eppendorf tube. In some cases incomplete destaining was noted.

Protein Alkylation

The excised, destained gel bands were equilibrated in 20 mM NaHCO₃ pH 7.0, for 2x30 min in eppendorf tubes. The last wash was replaced with 300 µl fresh 20 mM NaHCO₃ and

20 μ l 45 mM dithiothreitol (DTT) to reduce the protein at 60 °C for 30 min. Next, proteins were alkylated at room temperature in dark for 30 min., by the addition of 20 μ l 100 mM iodoacetamide. Residual SDS, DTT and iodoacetamide were removed after the final reaction by rinsing the gel pieces in acetonitrile: 20 mM NaHCO_3 pH 7.0 (1:1) 3x 45 min. Subsequently, the gel pieces were cut into smaller pieces using a clean glass plate, and dried in a rotary evaporator.

PNGaseF buffer exchange

PNGaseF was from Oxford GlycoSciences in a Tris HCl buffer, with EDTA and NaCl. However as Tris is difficult to remove before mass spectrometry, the buffer was exchanged to 20 mM NaHCO_3 pH 7.0 using Microcon (Amicon Inc.) microconcentrators.

40 units of PNGaseF in 80 μ l Tris buffer were made up to 400 μ l with 20 mM NaHCO_3 pH 7.0. This was applied to the microconcentrator and spinned at 14.000 g for 50 min. at 4°C. Another 500 μ l of NaHCO_3 pH 7.0 was added and spinned at 3000g for 50 min. at 4°C. The microconcentrator was then turned over and placed into a fresh eppendorf tube to collect the concentrated buffer exchanged PNGaseF after a quick spinning step. The PNGaseF recovered (in ~ 50 μ l volume) was then freeze dried, resuspended in 400 μ l 20 mM NaHCO_3 pH 7.0 (100U/ml) and used for further digestions as required.

In-gel enzymatic deglycosylation with PNGaseF

To the dried gel pieces 100 μ l (10U) of enzyme was added and incubated at 37°C overnight. In cases when gel bands have been pooled from two or more lanes, the incubation volume and enzyme concentration was increased appropriately to cover the swollen gel pieces.

Enzyme released glycan extraction

N-glycans released from the proteins were extracted by sonification in 200 μ l pure water 3x30 min. Washes and the incubation buffer were combined, incubated with 30 μ l of AG-50 (H^+) for 10 min., filtered and dried in rotary evaporator.

Fluorescent glycan labelling

Glycans were labelled with 2-aminobenzamide using the Signal Labeling Kit (Oxford GlycoSciences) following the manufacturer's instructions.

HPLC separation

N-linked 2-AB labelled glycans released and from fetuin were separated on GlycoSepC (Figure 12.3a) and from RNaseB on GlycoSepN (Figure 12.3b).

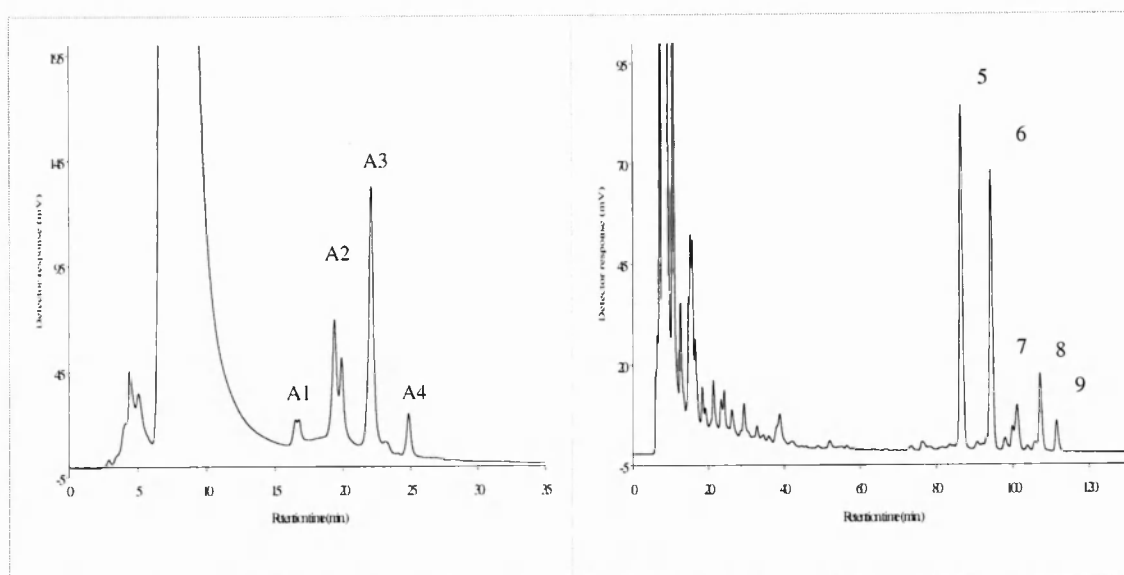


Figure 12.3. a.) GlycoSepC profile of the PNGaseF released N-glycans from fetuin (corresponding to 50 μ g protein). A1, A2, A3 and A4 are mono-, di-, tri- and tetrasialylated N-linked structures.

b.) GlycoSepN profile of RNaseB (~25 μ g protein). Numbers indicate the mannose residues in oligomannosidic structures.

As can be seen from the results on Figure 12.3, the method of Kuster et.al (1997) is a reproducible technique for the analysis of in-gel deglycosylated N-linked glycans from individual protein bands. All the N-linked glycans present on fetuin and RNaseB were

recovered in their correct proportions. Therefore the same procedure was used to analyse several of the chicken brain SPM glycoproteins separated by PAGE.

12.3 Glycan profiling of learning associated glycoprotein bands 33kDa, 45kDa and 180 kDa

The above described method was applied to a few chicken synaptic plasma membrane glycoproteins to analyse their N-linked glycosylation profile. The delipidated P2 fractions from MeA trained and quiet left IMHVs were separated on a 12.5% PAGE gel (Table 12.1 p.160), and 3 glycoprotein bands further analysed. For practical reasons, only one hemisphere and only three protein bands were analysed.

The glycoprotein bands of approx. molecular weights 33, 45 and 180 kDa were chosen on the basis of the results of Bullock et al. (1992). It has to be pointed out though, that in their study the lobus parolfactorius (LPO) region was analysed 6 and 24 hours after training and not the IMHVs 9 hours after training. However, those glycoproteins showed the most marked changes in fucose incorporation, therefore they were the most interesting candidates for further glycan analysis.

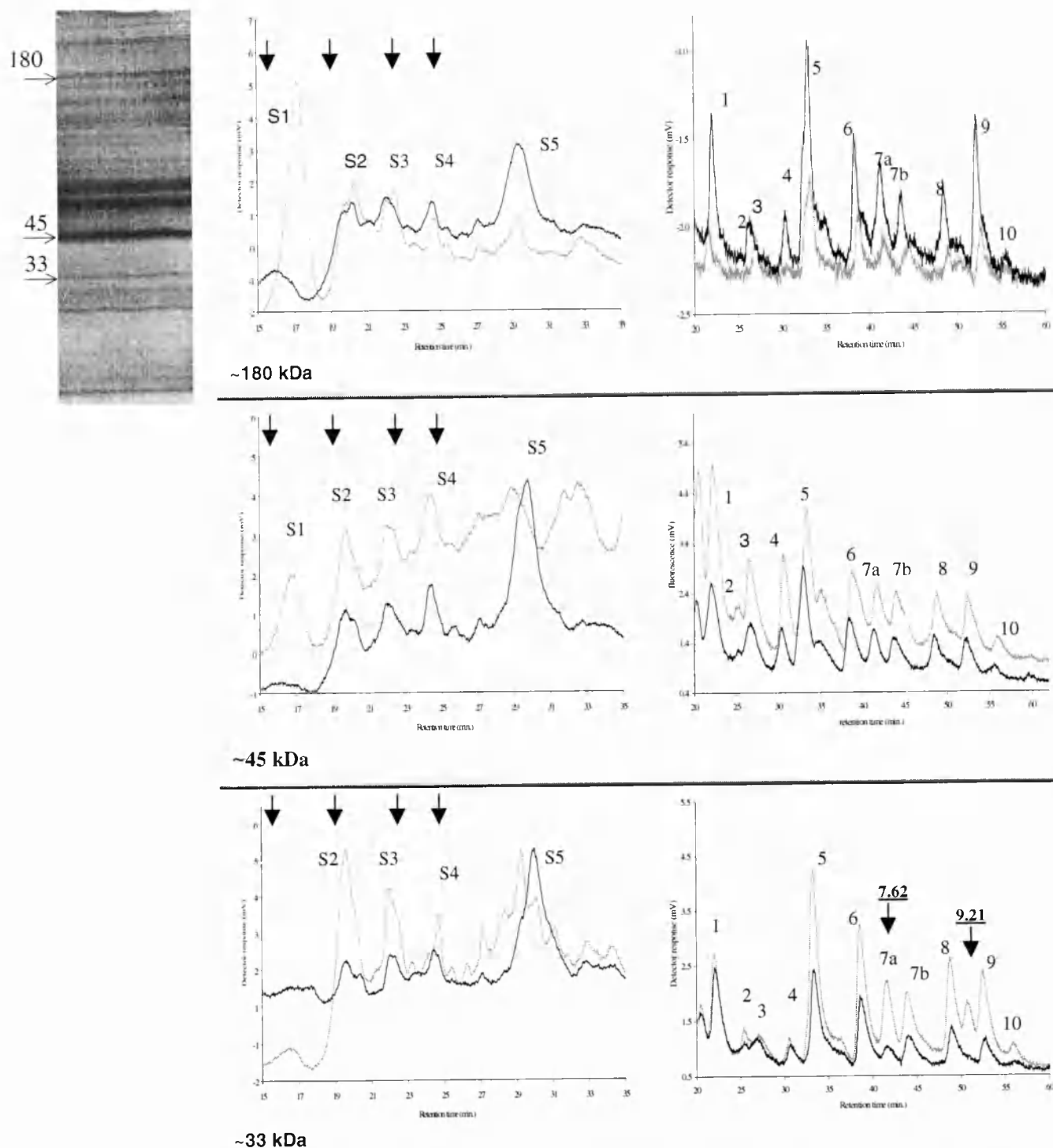


Figure 12.4. Weak anion-exchange (left panel) and normal-phase HPLC (right panel) of the N-linked glycans released from the 33 kDa, 45 kDa and 180 kDa left IMHV P2 fraction glycoproteins. Arrows on the left-panel indicate the elution position of mono- di- tri- and tetrasialylated glycans from fetuin (standard). S1-S4 are possible sialylated structures, the nature of highly negatively charged S5 is unknown. The arrows at the 33 kDa neutral profile point to the possible differences between the glycans of the same glycoprotein fraction from MeA trained vs. quiet animals. Dark line – quiet control, grey line- MeA trained.

Results

In contrast to the whole brain and IMHV glycan profiles, the prevailing majority (>90%) of the individual glycoprotein associated glycans were neutral.

The anionic glycans, peaks S2, S3 and S4, were present on all three of the glycoproteins analysed. Peak S1 (eluting between the monosialylated and disialylated fetuin standard), if present, could only be observed in MeA trained samples (180 kDa and 45 kDa). The identity and composition of the S5 peak is unknown, whereas peaks S1, S2, S3 and S4 are most probably sialylated glycans with 1,2,3 and 4 sialic acid residues, res.

The neutral profile presented on the right side panel is the naturally neutral glycan pool, which has been pooled during the weak-ion exchange chromatography.

The naturally neutral glycan profile is only shown from 20 minutes onward (~4 NPGU). The chromatogram is quite “noisy” between 0-20 minutes, but this area has been ignored on the basis of the assumption, that N-linked glycans are typically larger than 4 NPGU, and hence elute after 20 minutes.

180 kDa band

The **charged** profile of the 180 kDa component is characterised by the presence of a dominant, possibly monosialylated glycan in the MeA trained sample (S1). The **neutral** glycans contain the 5 possibly oligomannosidic structures (peaks 5-9). In addition, smaller NPGU peaks are also present, they could be hybrid glycans (peaks 1-4) or artefacts. No difference between the two samples can be observed, except the lower abundance of peak 4 in the MeA trained sample.

45 kDa band

The 45 kDa band is similar to the 180 kDa band, in the specific increase of the S1 charged peak in the trained sample only. Moreover, the unknown S5 peak is more abundant in the quiet control, similarly to the 180 kDa band. Peaks S2, S3 and S4 are very similar in both groups.

The neutral profile is dominated by the oligomannosidic glycans (peaks 5-9), however peaks 3 and 4 are more abundant then in the 180 kDa sample. Again, no training related differences seem to be present in the neutral profile.

33kDa

The 33 kDa glycoprotein is somewhat different from the previous two bands. The monosialylated structure is absent, however there is a dominant disialylated structure in the MeA sample, which is not so significant in the quiet group.

Even more interesting is probably the appearance of a novel neutral structure at **9.21** NPGU. This peak seems to be present only in the 33kDa band. A relative increase in peak **7a** is also evident in the trained sample.

Table 12.2 Normal phase glucose unit values of the neutral glycans separated on GlycoSepN (Figure 12.4) using the short gradient

Peak number	1	2	3	4	5	6	7a	7b	8	9	10
Identity					Man5 ?	Man6 ?	Man7 ?	Man7 ?	Man8 ?	Man9 ?	
NPGU	4.17	4.8	5.06	5.77	6.23	7.13	7.62	8.04	8.85	9.54	10.19

Discussion

Glycan profiling of the individual glycoprotein bands using the “in-gel deglycosylation” method has successfully separated a number of charged and neutral glycan species. The ratio of the charged to neutral glycans was markedly lower in the individual bands as compared to the same ratio of the IMHV glycans. This might be due to the differences in the glycan release procedure. PNGaseF has been used to release the N-linked glycans from

the protein bands, however it is possible, that (some of) the charged glycans could not be released by the enzyme.

Amongst the anionic oligosaccharides structures co-eluting with mono-, di-, tri- and tetrasialylated fetuin glycans were observed. The identity of these peaks could not be confirmed by sialidase digestion due to the low levels of sample material. However in future experiments it is possible to scale up the method without increasing the processing time. The same holds for the neutral glycan profile. The identity of peaks 5-9 cannot be stated with certainty, but they most probably represent the oligomannosidic glycans (Clark et al. 1998).

It is too early to make any conclusions about the observed differences between the two sample groups, though those peaks if real, are potentially extremely exciting. The method as applied to the chicken samples has **at least** one potential pitfall, which will have to be eliminated with additional experimentation. It is expected that from a complex gel profile as shown on Figure 12.1 p.159 single protein bands cannot be excised without impurities from neighbouring bands. A way around this problem might be to excise the target band from the first PAGE gel (e.g. the 33 kDa band) and to rerun this band on a higher % gel to achieve a better separation (i.e. zooming in on the band of interest). Even better would be to use partially purified starting material, or 2-dimensional electrophoresis.

In summary the method of Kuster et al. (1997) is potentially an extremely useful and easy-to-use method for analysing individual glycoproteins directly from PAGE gels. For conclusive results however the method requires relatively pure starting material, e.g. samples immunoprecipitated or partially purified.

12.4 *Lectin-blotting as a tool for analysing fucosylated glycoproteins*

Several studies indicated the presence of significant amounts of Ulex-binding or fucosylated glycoproteins in the SPM fractions of rat brain (Zanetta 1977, Pereira 1978, (Stanojev 1987)). No 2-AB labelled chicken brain glycans were bound to immobilised Ulex europaeus column. This was an unexpected finding, based on the previous studies with 2-deoxygalactose induced reduction in fucose incorporation, which also causes amnesia for the passive avoidance learning task. However, it is often noted that lectins may have different affinities depending whether they are soluble or immobilised. Moreover, the same glycan structure may be differentially recognized depending whether it is present on the glycoprotein or as a free oligosaccharide (e.g. 2-AB labelled). It was therefore reasoned that probably the glycan conformation is altered when the glycans are released from the glycoprotein, and that this altered conformation is not recognized by the lectin. Or, as it was mentioned previously, maybe these structures are relatively minor components.

Therefore as an alternative method to Ulex affinity chromatography, the in-gel deglycosylation method was used to analyse the glycan sequences of lectin positive glycoproteins as identified by lectin blotting of IMHV glycoproteins.

An overview of the literature using the Ulex lectin however indicated potential pitfalls (see next section). The experiments were also an attempt to resolve this controversy.

In addition to Ulex europaeus, two other fucose specific lectins were used: Anguilla anguilla and Lotus tetragonolobus.

Ulex-europaeus I was the first lectin studied. This is a widely used lectin with primary specificity for α 1-2 linked terminal fucose residues (Verbert 1995); the specificity of the soluble and immobilised lectin binding activity seems to be different, but has not been explored in detail (Verbert 1995). It is widely accepted, that UEA-I prefers Fuc α 1-2Gal β 1-4GlcNAc (H type 2 blood group antigen) structures, with some cross-reactivity with Lewis^y antigen (Fuc α 1-2Gal β 1-4(Fuc α 1-3)GlcNAc β).

Barber (1989) have used *Ulex europaeus* I for the fractionation of synaptosomal plasma membrane glycoproteins from rat brains, 26% of the synaptosomal proteins bound to the lectin column.

In a histochemical approach however, *Ulex* was found to recognise exclusively the primary olfactory neurons, and in the same study, a major 65 kDa glycoprotein on Western blots (Pestean et al. 1995).

A study by Zambenedetti et al. (1996) seemed to confirm the restricted expression of *Ulex* binding glycoproteins to the olfactory nerve and glomeruli of the olfactory bulb. However, affinity chromatography of the olfactory bulb glycoproteins on *Ulex* lectin column specifically bound a high Mw single glycoprotein, what the authors identified as N-CAM.

Systematic studies on the adult rat brain using an array of 31 lectins, did not detect any neuronal staining with *Ulex europaeus*, and the binding was only associated with oligodendrocytes (Zambenedetti et al. 1996).

Mayanil and Knepper (1993) followed the developmental changes of the synaptic plasma membrane associated carbohydrates in the mouse cerebral cortex, cerebellum and spinal cord during development. *Ulex* positive glycoproteins were only present in the synaptic vesicle fraction associated with a broad 85-100 kDa fraction.

In the paper by Bullock et al. (1992), and Yan et al. (1997), a large number of chicken SPM glycoproteins were reactive with the lectin.

The above representative list shows the possible pitfalls of the lectin affinity or lectin blotting methodology. The inconclusive results from a number of studies would suggest the use of lectins only as an additional research tool, and more rigorous carbohydrate analysis would be required to confirm preliminary conclusions.

Lotus tetragonolobus in contrast to UEA-I preferentially binds α 1-3 linked fucose residues in the Lewis-X motif, but not sialylated Lewis X (Baldus et al. 1996).

Anguilla anguilla agglutinin (AAA) has similar specificity to UEA-I, however, in addition it binds to H type 1 structures (Fuc α 1-2Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc) as well (Clark et al. 1998).

Results

Biotin-conjugated lectins were purchased from EY Laboratories, and used for lectin blotting of SPM glycoproteins as described in the Methods chapter (p.72). The glycoproteins reactive with the individual lectins are shown on Figure 12.5.

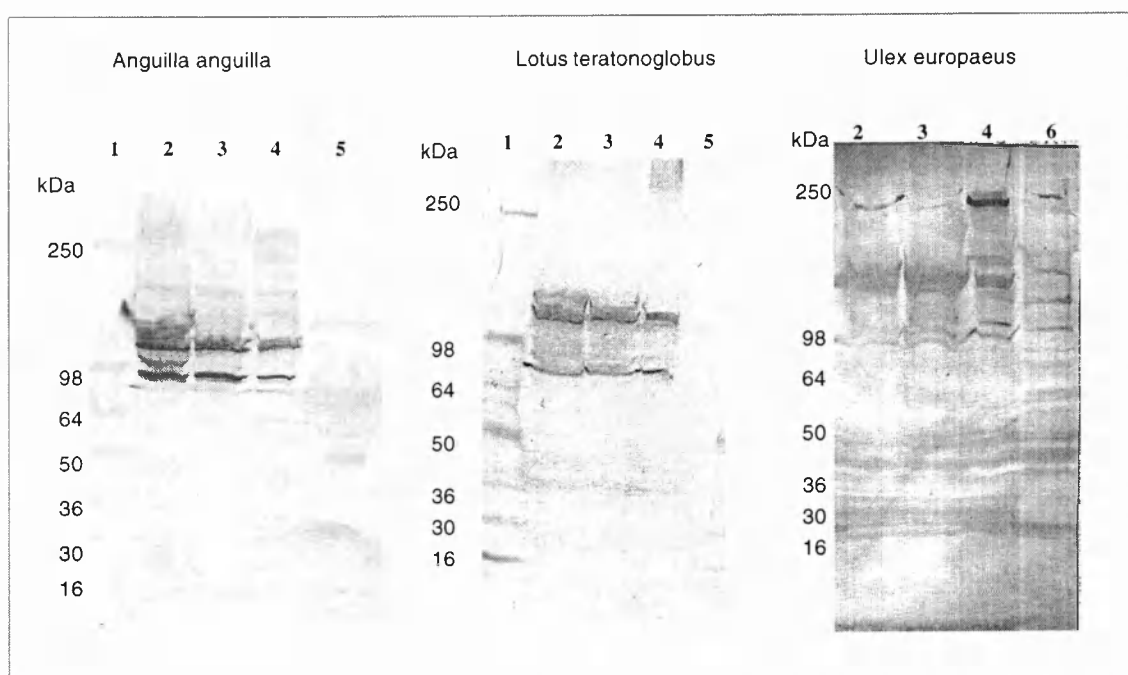


Figure 12.5. 1. Mw standard, 2. left-IMHV P2 fraction, 3. right-IMHV P2 fraction, 4. whole brain homogenate, 5. control fetuin, 6. HL60 cell line hydrolysate

Interestingly, only a limited number of lectin-reactive bands were observed even when non-gradient gels and different transfer conditions were used (data not shown). The bands of estimated M_w =85-90 kDa (Lotus bottom band), 110-120 kDa (AAA and Lotus top bands) and 220 kDa (Ulex top) were further analysed in order to determine the glycan structures associated with these bands. Left IMHV P2 fractions from trained and quiet chickens were chosen for further glycan characterisation.

The HPLC analysis of the 2-AB labelled N-linked glycans released from the lectin-reactive bands revealed that neutral structures predominate, because no charged glycans could be

detected by weak-ion exchange chromatography on GlycoSepC. The normal phase chromatographic profile indicated the presence of the five (most probably) oligomannosidic structures. However, as the chromatograms presented on Figure 12.6 represent 50% of the total glycan pool prepared from the PAGE gel bands, further investigation of these structures was not possible.

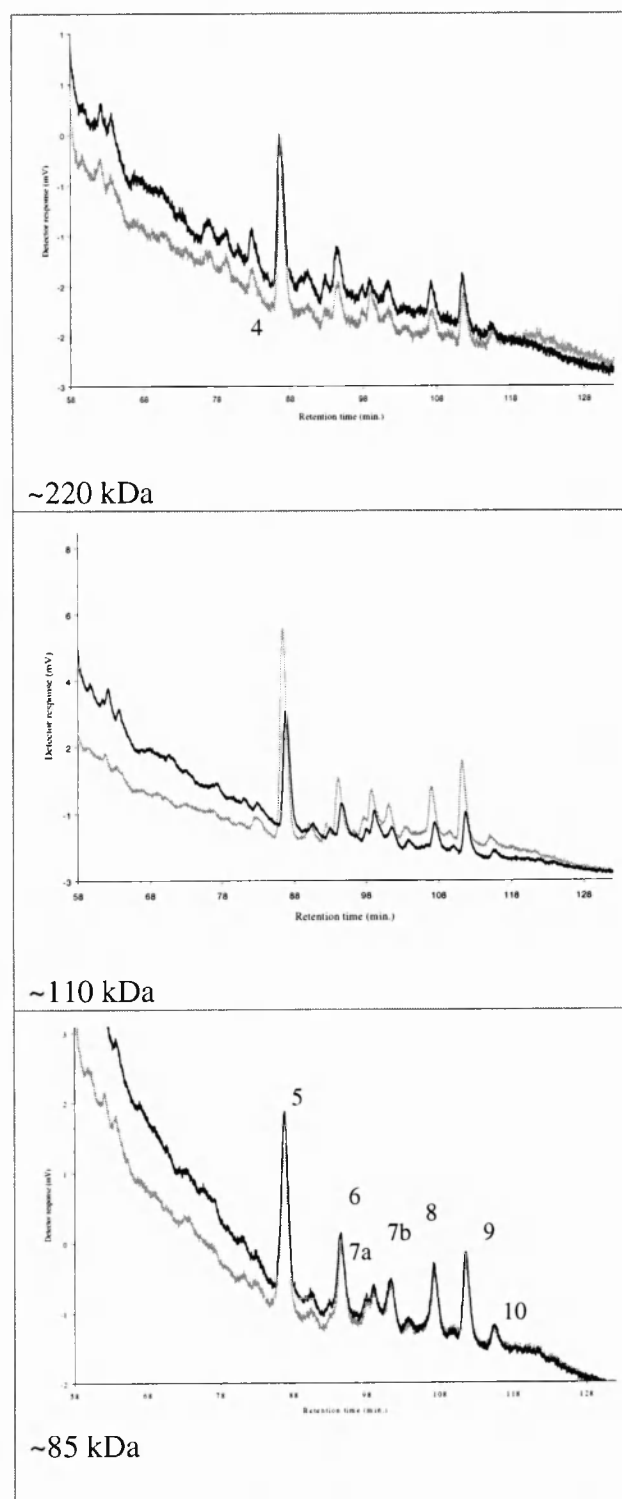


Figure 12.6 Normal phase HPLC of the neutral N-linked glycans associated with the IMHV P2 fraction glycoproteins: a) ~220 kDa Ulex-positive, b) ~110kDa Lotus and AAA positive, c) ~85 kDa Lotus positive band.

Table 12.3 Normal-phase glucose unit values of the N-linked glycans on Figure 12.6

Peak number	4	5 Man5 ?	6 Man6 ?	7a Man7 ?	7b Man7 ?	8 Man8 ?	9 Man9 ?	10 ?
NPGU (long gradient)	5.93	6.31	7.21	7.81	8.15	9.06	9.78	10.53

Discussion

The predominant glycan species (Man5-9) had normal phase glucose unit values (NPGU) corresponding to the oligomannosidic structures (Table 12.3), however peaks eluting at 5.93 and 10.53 NPGU have also been noted. Additional digestions with fucosidases or mannosidases and mass-spectrometric analysis would have been necessary to characterise the individual peaks, however the sample levels were too low to perform these digestions.

At this stage, the similarity of the neutral glycan profiles from 6 different glycoprotein bands (3 from the previous chapter) is striking. It is even more so, when compared to the neutral glycan profiles of the rat NMDA receptor R1 subunit and the AMPA receptor from the rat brain (Clark et al. 1998). It was expected, that individual glycoproteins would have (at least partially) characteristic glycan profiles. This is not immediately apparent from the neutral profiles, although it is still possible, that the profile of relatively minor structures may be unique to each individual band analysed.

13. Final discussion and future perspectives

The aims of this thesis were to identify the possible glycan structures into which the fucose is being incorporated in increased levels after training, and to characterise the chicken synaptic plasma membrane glycosylation profile.

Oligosaccharide analysis using chromatographic techniques in conjunction with fluorescent labelling was used to achieve these objectives.

The previously reported increases in fucose incorporation were 16-29% higher in the trained animals than in controls (both *in vivo* Sukumar et al. 1980 and *in vitro* McCabe and Rose 1985), therefore it was assumed that the glycan structures, where the fucose is being incorporated after training would be relatively straightforward to detect by comparing the glycan profiles from the trained and quiet animals.

The above mentioned precursor incorporation studies do not distinguish between the possibilities of increased turnover versus increased incorporation. However it was assumed that the increase in fucose incorporation was due to an increased glycan/glycoprotein synthesis and not due to increased turnover. This was based on the morphological evidence from the IMHVs and LPOs where an increased spine density and increased synaptic branching pattern can be observed after training. Such changes would require new synaptic membrane synthesis, and hence an increased synthesis of membrane glycoproteins.

Pure SPMs

In the first set of experiments, detergent extracted glycoproteins from pure synaptosomal plasma membranes (prepared from the forebrains) were compared from trained and quiet control animals. No difference between the two glycan profiles was evident, and this lack of difference was attributed to the sample preparation. It was thought, that if changes occur in relatively small brain regions, like the LPO and IMHV, analysis of the **whole** brain glycan pool might not be suitable for the detection of subtle changes restricted to those areas. Although no learning related changes were observed, the results from these

experiments have established the working conditions and some baseline information about the glycan composition of the chicken synaptic plasma membranes.

IMHV glycan analysis

In the second sample set a similar glycan comparison was performed however SPMs were prepared from the IMHVs* instead of the whole brain. Lateralisation of biochemical changes accompanying imprinting or passive avoidance training have been repeatedly noted (Rogers 1995), therefore hemispheric differences were also examined.

The charged profile from the IMHV preparation was different from the profile of pure SPMs. This could be due to several factors, perhaps the most important is the different extraction procedure used during the glycoprotein preparation from the membranous material. Another contributing factor might have been the difference between the fractionation procedures, pure SPM prep vs. P2 fraction. It cannot be excluded, that the charged material present only in the IMHV P2 fraction is due to contamination derived from glial cell membrane, mitochondria, myelin fragments etc. present in the P2 fraction. And finally, the reproducibility of the method is unknown. However the presence of a charged peak (eluting at 24.8 min) only in the left hemisphere (both trained and quiet) and the very similar charged and neutral glycan profiles from the corresponding hemispheres would indicate that the sample-to sample variation due to the methodology might have been relatively low. These are only suggestions, and further experiments would be required to clarify these questions.

As for the learning related changes, some differences were observed in the charged fraction, most notably in the right IMHV (p.130) and the individual glycoprotein bands analysed (p.164), however the unknown reproducibility of the method makes the interpretation of these findings difficult. It is, however, noteworthy that some differences

* IMHVs and LPOs should have been analysed, however due to the considerable processing time required by the analytical methods only IMHVs were analysed in this study.

in the charged fraction were noticed in three different experiments (IMHVs, individual gel bands and dark-hatched chicks). This may suggest an important role for this class of glycans, and it would be worthwhile to explore these glycans in more detail. Terminal charged glycan residues are known to be crucial in a large number of specific recognition and adhesion phenomena in the immune system. It would not be surprising if similar mechanisms were present in the nervous system as well.

Characterisation of the chicken SPM glycosylation profile

The second aim of this thesis was to characterise the synaptic plasma membrane associated glycoprotein glycans. The first unexpected finding was the presence of phosphorylated and sulphated glycans in addition to sialic acid in the negatively charged fraction. Generally sialic acid is considered to be the major negatively charged residue of glycoprotein glycans, however these results suggest the presence of other negatively charged residues, esp. phosphate and sulphate. These results seem to confirm previous data from mice brains where significant amounts of sulphated and phosphorylated glycans were described (Wing et al. 1992).

Glycosylation across species

Another aspect of the findings is the very similar neutral glycan profile from the adult rat, mouse and day-old chicken brains. The neutral glycan profiles on P4 are almost identical, despite considerable differences in age. The normal-phase HPLC profile was also very similar. Rat SPMs as well as individual glycoproteins from the rat brain, the NMDA receptor NR1 subunit and AMPA receptor (Mollicone et al. 1992) were comparable to the chicken SPM fractions and glycoprotein bands. At the level of individual peaks, the most common complex-type glycans are identical in the rat and chick, and there are indications that they might be the predominant structures in the human and mouse brain as well. The obvious implication would be that these structures might have a specific function in the brain. However D1 (p.149), the most abundant structure, is a bisecting glycan, and it was shown by Priatel et al. (1997) that mice deficient in GlcNAcT III (the enzyme synthesising

bisecting GlcNAc) show no obvious phenotypic consequences (p.58). It would be interesting to characterise the cognitive abilities of this mouse strain.

The second most abundant complex-type glycan structure (in rat, mouse and chicken) carries the LewisX epitope (p.150). The function and properties of this glycan epitope have been discussed in detail (LewisX epitope, p.58). It is expected that LewisX might be the most common fucosylated epitope in the chicken brain as well (Finne 1990; Chen et al. 1998), and therefore it would be very interesting to follow the activity of α 1-3 fucosyltransferase(s) after training on the passive avoidance learning task.

However the number, substrate specificity and distribution of different α 1-3 fucosyltransferases should also be followed, as it is known that different tissues typically express a mixture of different enzyme activities, which might be independently regulated (Schmidt 1995).

Another aspect of the LewisX containing glycoproteins is the identity –if exists- of the possible receptors for this oligosaccharide. It has been mentioned previously that LewisX displays homophilic adhesion in the presence of calcium and magnesium (p.58). Whether such interactions occur in the nervous system is not yet known. The idea, that changes in extracellular calcium are crucial during the learning process has been verified in a large number of experimental paradigms. That complex N-linked glycans may also be involved in this process is an interesting suggestion. (Shashoua 1991) described the unusual properties of the learning-related glycoprotein, ependymin. This small molecular weight extracellular glycoprotein contains two N-linked glycosylation sites, though the nature of the sugars is unknown, except for the high content of sialic acid and the presence of 3-sulphated glucuronic acid. Ependymin was found to be the second major calcium binding protein in the goldfish brain after calmodulin, and this calcium binding was mediated by the glycan structures present on ependymin. (Shashoua 1991) suggests, that change (drop) in the extracellular calcium concentrations at active synapses following learning induces the polymerisation of ependymin and stabilises the new conformation of the active

dendrite. Ependymin is also present in the mammalian brain and it appears to be involved in the consolidation step of learning (Shashoua 1991). This lucrative hypothesis has not yet been experimentally verified, nevertheless it points out the capacity of glycoprotein bound sugars to bind physiologically important metal ions, and the possible consequences of this binding on glycoprotein conformation.

In summary the aims of the thesis were relatively ambitious given the knowledge of chicken brain glycan composition that was available at the beginning of these studies. As a result, the experiments turned out to be a methodological enterprise toward the same goal, i.e. the identification of learning related glycan epitopes. Nevertheless interesting new data have been obtained as a result of several different experiments, which raise exciting new questions about the possible role of LewisX-containing structures and oligomannosidic glycans in the nervous system.

14. References

- Abeijon, C., Mandon, E. C., Hirschberg, C. B. (1997). "Transporters of nucleotide sugars, nucleotide sulfate and ATP in the Golgi apparatus." *Trends In Biochemical Sciences*, 22(6), 203-207.
- Ali, S. M., Bullock, S., and Rose, S. P. R. (1988). "Protein Kinase-C Inhibitors Prevent Long-Term-Memory Formation in the One-Day-Old Chick." *Neuroscience Research Communications*, 3(3), 133-140.
- Allendoerfer, K. L., Magnani, J. L., and Patterson, P. H. (1995). "Forse-1, an Antibody That Labels Regionally Restricted Subpopulations Of Progenitor Cells In the Embryonic Central-Nervous-System, Recognizes the Le(X) Carbohydrate On a Proteoglycan and 2 Glycolipid Antigens." *Molecular and Cellular Neuroscience*, 6(4), 381-395.
- Anokhin, K. V., Mileusnic, R., Shamakina, I. Y., and Rose, S. P. R. (1991). "Effects of Early Experience On C-Fos Gene-Expression in the Chick Forebrain." *Brain Research*, 544(1), 101-107.
- Anokhin, K. V., and Rose, S. P. R. (1991). "Learning-Induced Increase of Immediate Early Gene Messenger-RNA in the Chick Forebrain." *European Journal of Neuroscience*, 3(2), 162-167.
- Arami, S., Jucker, M., Schachner, M., and Welzl, H. (1996). "The effect of continuous intraventricular infusion of L1 and NCAM antibodies on spatial learning in rats." *Behav Brain Res*, 81(1-2), 81-7.
- Argandona, E. G., and Lafuente, J. V. (1996). "Effects of Dark-Rearing on the Vascularization of the Developmental Rat Visual-Cortex." *Brain Research*, 732(1-2), 43-51.
- Ashwell, K. W. S., and Mai, J. K. (1997a). "Developmental expression of the CD15 epitope in the hippocampus of the mouse." *Cell and Tissue Research*, 289(1), 17-23.
- Ashwell, K. W. S., and Mai, J. K. (1997b). "Developmental expression of the CD15-epitope in the brainstem and spinal cord of the mouse." *Anatomy and Embryology*, 196(1), 13-25.
- Ashwell, K. W. S., and Mai, J. K. (1997c). "A transient CD15 immunoreactive sling in the developing mouse cerebellum." *International Journal Of Developmental Neuroscience*, 15(7), 883-889.
- Ashwell, K. W. S., and Mai, J. K. (1997d). "Transient developmental expression of CD15 in the motor and auditory cortex of the mouse." *Developmental Brain Research*, 100(1), 143-148.
- Bahr, B. A., Staubli, U., Xiao, P., Chun, D., Ji, Z. X., Esteban, E. T., and Lynch, G. (1997). "Arg-Gly-Asp-Ser-selective adhesion and the stabilization of long-term potentiation: pharmacological studies and the characterization of a candidate matrix receptor." *J Neurosci*, 17(4), 1320-9.
- Bailey, C. H., Bartsch, D., and Kandel, E. R. (1996). "Toward a molecular definition of long-term memory storage." *Proc. Natl. Acad. Sci.*, 93, 13445-13452.
- Baldus, S. E., Thiele, J., Park, Y. O., Hanisch, F. G., Bara, J., and Fischer, R. (1996). "Characterization of the Binding-Specificity of Anguilla-Anguilla Agglutinin (AAA) in Comparison to Ulex-Europaeus Agglutinin-I (UEA-I)." *Glycoconjugate Journal*, 13(4), 585-590.

- Barber, P. C. (1989). "Ulex-Europeus Agglutinin-I Binds Exclusively to Primary Olfactory Neurons in the Rat Nervous-System." *Neuroscience*, 30(1), 1-9.
- Baudry, M., Thompson, R. F., and Davis, J. L. (1993). *Synaptic Plasticity*, MIT Press, Cambridge, MA.
- Bayard, B., Roux, D. (1975). "Hydrazinolysis and nitrous deamination of glycoproteins. Evidence for a common inner core in a carbohydrate moiety." *FEBS Letters*, 55, 206-211.
- Becker, C. G., Artola, A., Gerardyschahn, R., Decker, T., Welzl, H., and Schachner, M. (1996). "The Polysialic Acid Modification of the Neural Cell-Adhesion Molecule Is Involved in Spatial-Learning and Hippocampal Long-Term Potentiation." *Journal of Neuroscience Research*, 45(2), 143-152.
- Beesley, P. W., Mummery, R., and Tibaldi, J. (1995). "N-cadherin is a major glycoprotein component of isolated rat forebrain densities." *J. Neurochem.*, 64, 2288-2294.
- Benevento, L. A., Bakkum, B. W., Port, J. D., and Cohen, R. S. (1992). "The effects of dark-rearing on the electrophysiology of the rat visual cortex." *Brain Res*, 572(1-2), 198-207.
- Bergeron, J. J. M., Zapun, A., Ou, W.-J., Hemming, R., Parlati, F., Cameron, P.H., Thomas, D.Y. (1997). "The role of the lectin calnexin in conformation independent binding to N-linked glycoproteins and quality control." *Glycoimmunology 2*, J. S. Axford, ed., Plenum Press, 105-116.
- Binns, K. E., Withington, D. J., and Keating, M. J. (1995). "The Developmental Emergence of the Representation of Auditory Azimuth in the External Nucleus of the Inferior Colliculus of the Guinea-Pig - the Effects of Visual and Auditory Deprivation." *Developmental Brain Research*, 85(1), 14-24.
- Bliss, T. V. P., and Collingridge, G. L. (1993). "A synaptic model of memory: long-term potentiation in the hippocampus." *Nature*, 361, 31-39.
- Bliss, T. V. P., and Lomo, T. (1973). "Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path." *J. Physiol.*, 232, 331-356.
- Boubelik, M., Floryk, D., Bohata, L., Macak, J., and Craber, P. (1998). "LeX glycosphingolipids-mediated cell aggregation." *Glycobiology*, 8(2), 139-146.
- Bretscher, M. S., and Munro, S. (1993). "Cholesterol and the Golgi apparatus." *Science*, 261(5126), 1280-1.
- Brown, D. F. (1984). "The role of adenosine 3',5'-cyclic monophosphate (cAMP) in memory formation: A behavioral pharmacological, and biochemical study," Ph.D, La Trobe University.
- Bruses, J. L., and Rutishauser, U. (1998). "Regulation of neural cell adhesion molecule polysialylation: Evidence for nontranscriptional control and sensitivity to an intracellular pool of calcium." *Journal of Cell Biology*, 140(5), 1177-1186.
- Bullock, S., Degraan, P. N. E., Oestreicher, A. B., Gispen, W. H., and Rose, S. P. R. (1990). "Identification of a 52 kDa Chick Brain Membrane-Protein Showing Changed Phosphorylation after Passive-Avoidance Training As B-50 (Gap-43)." *Neuroscience Research Communications*, 6(3), 181-186.
- Bullock, S., Rose, S. P. R., and Zamani, R. (1992). "Characterization and Regional Localization of Presynaptic and Postsynaptic Glycoproteins of the Chick Forebrain Showing Changed Fucose Incorporation Following Passive-Avoidance Training." *Journal of Neurochemistry*, 58(6), 2145-2154.
- Bunnell, B. A., Adams, D. E., and Kidd, V. J. (1990). "Transient Expression of a P58 Protein-Kinase cDNA Enhances Mammalian Glycosyltransferase Activity." *Biochemical and Biophysical Research Communications*, 171(1), 196-203.

- Burchuladze, R., Potter, J., and Rose, S. P. R. (1990). "Memory Formation in the Chick Depends On Membrane-Bound Protein- Kinase-C." *Brain Research*, 535(1), 131-138.
- Burgoyne, R. D., and Rose, S. P. (1980). "Subcellular localization of increased incorporation of [3H]fucose following passive avoidance learning in the chick." *Neurosci Lett*, 19(3), 343-8.
- Bussolino, D. F., Guido, M. E., and Caputto, B. L. (1997). "Light exposure stimulates the activity of ganglioside glycosyltransferases of retina ganglion cells." *Neurochemistry International*, 31(1), 105-111.
- Caputto, B. L., Nores, G. A., Cemborain, B. N., and Caputto, R. (1982). "The effect of light exposure following an intraocular injection of [3H]N-acetylmannosamine on the labeling of gangliosides and glycoproteins of retina ganglion cells and optic tectum of singly caged chickens." *Brain Res*, 245(2), 231-8.
- Carlson, D. M. (1968). "Structures and immunochemical properties of oligosaccharides isolated from pig submaxillary mucins." *J Biol Chem*, 243(3), 616-26.
- Catalano, S. M., Chang, C. K., and Shatz, C. J. (1997). "Activity-dependent regulation of NMDAR1 immunoreactivity in the developing visual cortex." *Journal of Neuroscience*, 17(21), 8376-8390.
- Chang, F.-L. F., and Greengough, W. T. (1984). "Transient and enduring morphological correlates of synaptic activity and efficacy change in the rat hippocampal slice." *Brain Research*, 309, 35-46.
- Chen, Y. J., Wing, D. R., Guile, G. R., Dwek, R. A., Harvey, D. J., and Zamze, S. (1998). "Neutral N-glycans in adult rat brain tissue - Complete characterisation reveals fucosylated hybrid and complex structures." *European Journal Of Biochemistry*, 251(3), 691-703.
- Cherkin, A. (1969). "Kinetics of memory consolidation: role of amnestic treatment parameters." *Proc. Natl. Acad. Sci.*, 63, 1094-1101.
- Childs, R. A., Pennington, J., Uemura, K., Scudder, P., Goodfellow, P. N., Evans, M. J., and Feizi, T. (1983). "High-molecular-weight glycoproteins are the major carriers of the carbohydrate differentiation antigens I, i and SSEA-1 of mouse teratocarcinoma cells." *Biochem. J.*, 215, 491-503.
- Chothia, C., and Jones, Y. E. (1997). "The molecular structure of cell adhesion molecules." *Ann. Rev. Biochem.*, 66, 823-862.
- Chou, D. K. H., and Jungalwala, F. B. (1993). "Characterization and Developmental Expression of a Novel Sulfotransferase For the Biosynthesis of Sulfoglucuronyl Glycolipids in the Nervous-System." *Journal of Biological Chemistry*, 268(1), 330-336.
- Chou, D. K. H., and Jungalwala, F. B. (1996). "N-Acetylglucosaminyl Transferase Regulates the Expression of the Sulfoglucuronyl Glycolipids in Specific Cell-Types in Cerebellum During Development." *Journal of Biological Chemistry*, 271(46), 28868-28874.
- Clark, R. A. C., Gurd, J. W., Bissoon, N., Tricaud, N., Molnar, E., Zamze, S. E., Dwek, R. A., McIlhinney, R. A. J., and Wing, D. R. (1998). "Identification of lectin-purified neural glycoproteins, GPs 180, 116, and 110, with NMDA and AMPA receptor subunits: Conservation of glycosylation at the synapse." *Journal of Neurochemistry*, 70(6), 2594-2605.
- Clements, M. P., and Rose, S. P. R. (1996). "Time-Dependent Increase in Release of Arachidonic-Acid Following Passive-Avoidance Training in the Day-Old Chick." *Journal of Neurochemistry*, 67(3), 1317-1323.

- Cole, G. J., and McCabe, C. F. (1991). "Identification of a Developmentally Regulated Keratan Sulfate Proteoglycan That Inhibits Cell Adhesion and Neurite Outgrowth." *Neuron*, 7, 1007-1018.
- Cook, W. J., and Bugg, C. E. (1975). "Calcium-carbohydrate bridges composed of uncharged sugars. Structure of a hydrated calcium bromide complex of alpha-fucose." *Biochim Biophys Acta*, 389(3), 428-35.
- Coughlan, C. M., Seckl, J. R., Fox, D. J., Unsworth, R., and Breen, K. C. (1996). "Tissue-Specific Regulation of Sialyltransferase Activities In the Rat By Corticosteroids In-Vivo." *Glycobiology*, 6(1), 15-22.
- Cremer, H., Lange, R., Christoph, A., Plomann, M., Vopper, G., Roes, J., Brown, R., Baldwin, S., Kraemer, P., Scheff, S., Barthels, D., Rajewsky, K., and Wille, W. (1994). "Inactivation of the N-CAM Gene in Mice Results in Size-Reduction of the Olfactory-Bulb and Deficits in Spatial-Learning." *Nature*, 367(6462), 455-459.
- Crimaudo, C. H., M.; Gausepohl, H.; Meyer, D.;. (1987). "Human ribophorins I and II: the primary structure and membrane topology of two highly conserved endoplasmic reticulum-specific glycoproteins." *EMBO Journal*, 6, 75-82.
- Crowe, S. F., Zhao, W. Q., Sedman, G. L., and Ng, K. T. (1994). "2-deoxygalactose interferes with an intermediate processing stage of memory." *Behav Neural Biol*, 61(3), 206-13.
- Czepita, D., Reid, S. N., and Daw, N. W. (1994). "Effect of longer periods of dark rearing on NMDA receptors in cat visual cortex." *J Neurophysiol*, 72(3), 1220-6.
- Daisley, J. N., and Rose, S. P. R. (1994). "The Effect of a Passive-Avoidance Task On the Release of Amino-Acids in-Vitro From the Left Intermediate Medial Hyperstriatum Ventrale (IMHV) of the Day-Old Chick." *Biochemical Society Transactions*, 22(2), S 160-S 160.
- Desmond, N. L., and Levy, W. B. (1986). "Changes in the postsynaptic density with long-term potentiation in the dentate gyrus." *Journal of Comparative Neurology*, 253, 476-482.
- Desruisseau, S., Valette, A., Franc, J. L., and Chabaud, O. (1996). "Thyrotropin Controls Dolichol-Linked Sugar Pools and Oligosaccharyltransferase Activity In Thyroid-Cells." *Molecular and Cellular Endocrinology*, 122(2), 223-228.
- DeZazzo, J., and Tully, T. (1995). "Dissection of memory formation:from behavioral pharmacology to molecular genetics." *TINS*, 18, 212-218.
- Dontenwill, M., Roussel, G., Zanetta, J-P. (1985). "Immunohistochemical localization of a lectin like molecule R1 during the postnatal development of the rat cerebellum." *Dev. Brain Res.*, 17, 245-252.
- Doubell, T. P., and Stewart, M. G. (1993). "Short-Term Changes in the Numerical Density of Synapses in the Intermediate and Medial Hyperstriatum-Ventrale Following One-Trial Passive-Avoidance Training in the Chick." *Journal of Neuroscience*, 13(5), 2230-2236.
- Doyle, E., Nolan, P. M., Bell, R., and Regan, C. M. (1992a). "Hippocampal-NCAM180 Transiently Increases Sialylation During the Acquisition and Consolidation of a Passive-Avoidance Response in the Adult-Rat." *Journal of Neuroscience Research*, 31(3), 513-523.
- Doyle, E., Nolan, P. M., Bell, R., and Regan, C. M. (1992b). "Intraventricular Infusions of Antineural Cell-Adhesion Molecules in a Discrete Posttraining Period Impair Consolidation of a Passive- Avoidance Response in the Rat." *Journal of Neurochemistry*, 59(4), 1570-1573.
- Dudai, Y. (1989). *The Neurobiology of Memory Concepts, Findings, Trends*, Oxford University Press, New York.

- Eckhardt, M., Muhlenhoff, M., Bethe, A., Koopman, J., Frosch, M., and Gerardyschahn, R. (1995). "Molecular Characterization of Eukaryotic Polysialyltransferase-1." *Nature*, 373(6516), 715-718.
- Edelman, G. M., and Jones, F. S. (1998). "Gene regulation of cell adhesion: a key step in neural morphogenesis." *Brain Research Reviews*, 26, 337-352.
- Edge, C. J., Rademacher, T.W., Wormald, M.R., Parekh, R.B., Butters, T.D., Wing, D.R., Dwek, R.A. (1992). "Fast sequencing of oligosaccharides: The reagent-array nanalysis method." *Proc.Natl. Acad. Sci. USA*, 89, 6338-6342.
- Eggens, I., Fenderson, B., Toyokuni, T., Dean, B., Stroud, M., and Hakomori, S. (1989). "Specific Interaction between LeX and LeX Determinants." *J. Biol. Chem.*, 264, 9476-9484.
- Elbein, A. D. (1987). "Inhibitors of the biosynthesis and processing of N-linked oligosaccharide chains." *Ann. Rev. Biochim.*, 56, 497-534.
- Fagiolini, M., Pizzorusso, T., Porciatti, V., Cenni, M., and Maffei, L. (1997). "Transplant of Schwann cells allows normal development of the visual cortex of dark-reared rats." *European Journal of Neuroscience*, 9(1), 102-112.
- Fahrig, T., Schmitz, B., Weber, D., Kucherer-Ehret, A., Faissner, A., and Schachner, M. (1990). "Two monoclonal antibodies recognizing carbohydrate epitopes on neural cell adhesion molecules interfere with cell interactions." *Eur. J. Neurosci.*, 2, 153-161.
- Finne, J. (1982a). "Occurrence of Unique Polysialosyl Carbohydrate Units in glycoproteins of Developing Brain." *J. Biol. Chem.*, 257(October 25), 11966-11970.
- Finne, J., Krusius, T. (1982b). "Preparation and Fractionation of Glycopeptides." *Methods in Enzymology*, 269-277.
- Finne, J. (1990). "The Carbohydrate Units of Nervous Tissue Glycoproteins: Structural Properties and Role in Cell Interactions." *Morphoregulatory Molecules*, C. B. Edelman GM, Thiery JP, ed., John Wiley & Sons, 81-116.
- Finne, J., Krusius, T., and Rauvala, H. (1977). "Occurrence of disialosyl groups in glycoproteins." *Bioche. Biophys. Res. Commun.*, 74, 405-410.
- Flexner, L. B., and Flexner, J. B. (1966). "Effect of acetoxycycloheximide and of an acetoxycycloheximide-puromycin mixture on cerebral protein." *Proc. Natl. Acad. Sci.*, 55, 369-374.
- Freeman, F. M., Rose, S. P. R., and Scholey, A. B. (1995). "2 Time Windows of Anisomycin-Induced Amnesia for Passive-Avoidance Training in the Day-Old Chick." *Neurobiology of Learning and Memory*, 63(3), 291-295.
- Fukuda, M. (1994). *Molecular Glycobiology*, Oxford University Press, New York.
- Gocht, A., Struckhoff, G., and Lohler, J. (1996). "CD15-Containing Glycoconjugates in the Central-Nervous-System." *Histology and Histopathology*, 11(4), 1007-1028.
- Gooi, H. C., Feizi, T., Kapadia, A., Knowles, B. B., Solter, D., and Evans, M. J. (1981). "Stage-Specific Embryonic Antigen Involves Alpha-1-3 Fucosylated Type-2 Blood-Group Chains." *Nature*, 292(5819), 156-158.
- Gordon, B., Kinch, G., Kato, N., Keele, C., Lissman, T., and Fu, L. N. (1997). "Development of MK-801, kainate, AMPA, and muscimol binding sites and the effect of dark rearing in rat visual cortex." *Journal of Comparative Neurology*, 383(1), 73-81.
- Griffith, L. S., Mathes, M., and Schmitz, B. (1995). "Beta-Amyloid Precursor Protein Is Modified With O-Linked N- Acetylglucosamine." *Journal of Neuroscience Research*, 41(2), 270-278.

- Griffith, L. S., and Schmitz, B. (1995). "O-Linked N-Acetylglucosamine Is Up-Regulated in Alzheimer Brains." *Biochemical and Biophysical Research Communications*, 213(2), 424-431.
- Griffith, L. S., Schmitz, B., and Schachner, M. (1992). "L2/HNK-1 Carbohydrate and Protein-Protein Interactions Mediate the Homophilic Binding of the Neural Adhesion Molecule-P0." *Journal of Neuroscience Research*, 33(4), 639-648.
- Grover, L. M. (1998). "Evidence for postsynaptic induction and expression of NMDA receptor independent LTP." *J Neurophysiol*, 79(3), 1167-82.
- Guile, G. R., Rudd, P. M., Wing, D. R., Prime, S. B., and Dwek, R. A. (1996). "A Rapid High-Resolution High-Performance Liquid-Chromatographic Method for Separating Glycan Mixtures and Analyzing Oligosaccharide Profiles." *Analytical Biochemistry*, 240(2), 210-226.
- Gurd, J. W. (1989). *Neurobiology of Glycoconjugates*, Plenum Press, New York, 219-242.
- Hampel, H., Schwartz, M. J., Kotter, H. U., Schneider, C., and Muller, N. (1996). "Cell Adhesion Molecules in the Central Nervous System." *DN&P*, 9(2), 69-81.
- Hansen, J. E., Lund, O., Engelbrecht, J., Bohr, H., Nielsen, J. O., Hansen, J. E. S., and Brunak, S. (1997). "Prediction of O-Glycosylation Of Mammalian Proteins - Specificity Patterns Of UDP-GalNAc-Polypeptide N-Acetylgalactosaminyltransferase." *Biochemical Journal*, 308(Pt3), 801-813.
- Harland, C. E. (1994). "The Development of Organic Ion Exchange resins." *Ion exchange - Theory and Practice*, The Royal Society of Chemistry, Cambridge, 285.
- Hart, G. W., Greis, K. D., Dong, L. Y. D., Blomberg, M. A., Chou, T. Y., Jiang, M. S., Roquemore, E. P., Snow, D. M., Kreppel, L. K., Cole, R. C., and Hayes, B. K. (1995). "Ubiquitous and Temporal Glycosylation of Nuclear and Cytoplasmic Proteins." *Pure and Applied Chemistry*, 67(10), 1637-1645.
- Hart, G. W., Kreppel, L. K., Comer, F. I., Arnold, C. S., Snow, D. M., Ye, Z. Y., Cheng, X. G., Dellamanna, D., Caine, D. S., Earles, B. J., Akimoto, Y., Cole, R. N., and Hayes, B. K. (1996). "O-GlcNAcylation of Key Nuclear and Cytoskeletal Proteins - Reciprocity with O-Phosphorylation and Putative Roles in Protein Multimerization." *Glycobiology*, 6(7), 711-716.
- Hase, S. (1994). "High-performance Liquid Chromatography of Pyridylaminated Saccharides." *Guides to Techniques in Glycobiology*, W. J. Lennarz, Hart, G.W., ed., Academic Press, San Diego, 225-237.
- Hebb, D. O. (1949). *The Organization of Behaviour*, WILEY, New York.
- Hirschberg, C. B. (1997). "Transporters of nucleotide sugars, nucleotide sulfate and ATP in the Golgi apparatus membrane: Where next?" *Glycobiology*, 7(2), 169-171.
- Hoffmann, A., Nimtz, M., Getzlaff, R., and Conradt, H. S. (1995). "Brain-Type N-Glycosylation of Asialo-Transferrin From Human Cerebrospinal-Fluid." *Febs Letters*, 359(2-3), 164-168.
- Hoffmann, A., Nimtz, M., Wurster, U., and Conradt, H. S. (1994). "Carbohydrate Structures of Beta-Trace Protein from Human Cerebrospinal-Fluid - Evidence For Brain-Type N-Glycosylation." *Journal of Neurochemistry*, 63(6), 2185-2196.
- Holscher, C., and Rose, S. P. R. (1992). "An Inhibitor of Nitric-Oxide Synthesis Prevents Memory Formation in the Chick." *Neuroscience Letters*, 145(2), 165-167.
- Holscher, C., and Rose, S. P. R. (1994). "Inhibitors of Phospholipase a(2) Produce Amnesia For a Passive- Avoidance Task in the Chick." *Behavioral and Neural Biology*, 61(3), 225-232.
- Horn, G. (1991). "Techniques for removing IMHV from the chick brain." *Neural and Behavioural Plasticity. The use of the domestic chick as a model*, R. J. Andrew, ed., Oxford University Press, Oxford, 570.

- Horstkorte, R., Schachner, M., Magyar, J.P., Vorherr, T., Schmitz, B. (1993). "The fourth immunoglobulin-like domain of N-CAM contains a carbohydrate recognition domain for oligomannosidic glycans implicated in association with L1 and neurite outgrowth." *J. Cell Biol.*, 121, 1409-1421.
- Hunter, A., and Stewart, M. G. (1993). "Long-term increases in the numerical density of synapses in the chick lobus parolfactorius after passive avoidance training." *Brain Res*, 605(2), 251-5.
- Ihara, Y., Sakamoto, Y., Mihara, M., Shimizu, K., and Taniguchi, N. (1997). "Overexpression of N-acetylglucosaminyltransferase III disrupts the tyrosine phosphorylation of Trk with resultant signaling dysfunction in PC12 cells treated with nerve growth factor." *Journal of Biological Chemistry*, 272(15), 9629-9634.
- Ioffe, E., Liu, Y., and Stanley, P. (1997). "Complex N-glycans in Mgat1 null preimplantation embryos arise from maternal Mgat1 RNA." *Glycobiology*, 7(7), 913-919.
- Jacob, G. S., Kirmaier, C., Abbas, S. Z., Howard, S. C., Steininger, C. N., Welply, J. K., and Scudder, P. (1995). "Binding of sialyl Lewis X to E-selectin as measured by fluorescence polarization." *Biochemistry*, 34(4), 1210-7.
- Jentoft, N. (1990). "Why Are Proteins O-Glycosylated." *Trends in Biochemical Sciences*, 15(8), 291-294.
- Jibu, M., Pribram, K. H., and Yasue, K. (1996). "From conscious experience to memory storage and retrieval: the role of quantum brain dynamics and boson condensation of evanescent photons." *International Journal of Modern Physics B*, 10(13&14), 1735-1754.
- Johnston, A. N., Rogers, L. J., and Dodd, P. R. (1995). "[H-3] Mk-801 Binding Asymmetry in the IMHV Region of Dark-Reared Chicks Is Reversed By Imprinting." *Brain Research Bulletin*, 37(1), 5-8.
- Johnston, A. N. B., Clements, M. P., and Rose, S. P. R. (1998). "Role of brain-derived neurotrophic factor and presynaptic proteins in passive avoidance learning in day-old domestic chicks." *Neuroscience*, submitted.
- Jones, D. G., and Matus, A. I. (1974). "Isolation of synaptic plasma membrane from brain by combined flotation-sedimentation density gradient centrifugation." *Biochim. Biophys. Acta*, 356, 276-287.
- Jones, L. S. (1996). "Integrins: possible functions in the adult CNS." *TINS*, 19(2), 68-72.
- Jork, R., Grecksch, G., and Matthies, H. (1986). "Impairment of Glycoprotein Fucosylation in Rat Hippocampus and the Consequences on Memory Formation." *Pharmacology Biochemistry and Behavior*, 25, 1137-1144.
- Jork, R., Potter, J., Bullock, S., Grecksch, G., Matthies, H., and Rose, S. P. R. (1989). "Identification of Rat Hippocampal Glycoproteins Showing Changed Fucosylation Following 2-Deoxy-D-Galactose-Induced Amnesia in a Brightness-Discrimination Task." *Neuroscience Research Communications*, 5(2), 105-110.
- Takehi, K., Honda, S. (1993). "Analysis of Carbohydrates in Glycoproteins by High-Performance Liquid Chromatography and High-Performance Capillary Electrophoresis." *Glycoprotein Analysis in Biomedicine*, J.M.Walker, ed., Humana Press, Totowa, 81-98.
- Kamiguchi, H., and Lemmon, V. (1998). "A neuronal form of the cell adhesion molecule L1 contains a tyrosine-based signal required for sorting to the axonal growth cone." *Journal of Neuroscience*, 18(10), 3749-3756.
- Keller, P., and Simons, K. (1997). "Post-Golgi biosynthetic trafficking." *Journal of Cell Science*, 110(Pt24), 3001-3009.
- Kelm, G., Pelz, A., Schauer, R., Filbin, M. T., Tang, S., deBellard, M., Schnaar, R. L., Mahoney, J. A., Hartnell, A., Bradfield, P., and Crocker, P. R. (1994).

- "Sialoadhesin, myelin-associated glycoprotein and CD22 define a new family of sialic acid-dependent adhesion molecules of the immunoglobulin superfamily." *Current Biology*, 4(11), 965-972.
- Kelm, S., Schauer, R., and Crocker, P. R. (1996). "The Sialoadhesins - a Family of Sialic Acid-Dependent Cellular Recognition Molecules Within the Immunoglobulin Superfamily." *Glycoconjugate Journal*, 13(6), 913-926.
- Kiss, J. Z., and Rougon, G. (1997). "Cell biology of polysialic acid." *Current Opinion in Neurobiology*, 7(5), 640-646.
- Kojima, N., Fenderson, B. A., Stroud, M. R., Goldberg, R. I., Habermans, R., Tpyokuni, T., and Hakomori, S. (1994). "Further studies on cell adhesion based on LeX-LeX interaction, with new approaches: embryoglycan aggregation of F9 teratocarcinoma cells, and adhesion of various tumor cells based on LeX expression." *Glycoconjugate J.*, 11, 238-248.
- Kojima, N., Yoshida, Y., Kurosawa, N., Lee, Y. C., and Tsuji, S. (1995). "Enzymatic-Activity of a Developmentally-Regulated Member of the Sialyltransferase Family (STX) - Evidence For Alpha-2,8- Sialyltransferase Activity Toward N-Linked Oligosaccharides." *Febs Letters*, 360(1), 1-4.
- Kornfeld, R., Kornfeld, S. (1985). "Assembly of asparagine-linked oligosaccharides." *Ann. Rev. Biochem*, Annual Reviews Inc, 631-664.
- Kossut, M., and Rose, S. P. R. (1984). "Differential 2-Deoxyglucose Uptake into Chick Brain Structures During Passive-Avoidance Training." *Neuroscience*, 12(3), 971-977.
- Krug, M., Wagner, M., Staak, S., and Smalla, K.-H. (1994). "Fucose and fucose-containing sugar epitopes enhance hippocampal long-term potentiation in the freely moving rat." *Brain Research*, 643, 130-135.
- Krusius, T., Finne, J. (1977). "Structural features of tissue glycoproteins." *Eur. J. Biochem.*, 78, 369-379.
- Krusius, T., Finne, J. (1978a). "Characterization of a novel sugar sequence from rat-brain glycoproteins containing fucose and sialic acid." *Eur. J. Biochem.*, 84, 395-403.
- Krusius, T., Finne, J., Margolis, R.U., Margolis, R.K. (1978b). "Structural Features of Microsomal, Synaptosomal, Mitochondrial, and Soluble Glycoproteins of Brain." *Biochemistry*, 17(18), 3849-3854.
- Kucherer, A., Faissner, A., and Schachner, M. (1987). "The novel Carbohydrate epitope L3 is Shared by Some Neural Cell Adhesion Molecules." *J. Cell Biol.*, 104, 1597-1602.
- Kunemund, V., Jungalwala, F. B., Fischer, G., Chou, D. K. H., Keilhauer, G., and Schachner, M. (1988). "The L2/HNK-1 Carbohydrate of Neural Cell Adhesion Molecules is Involved in Cell Interactions." *J. Cell Biol.*, 106, 213-223.
- Kuster B, W. S., Hunter AP, Dwek RA, Harvey DJ. (1997). "Sequencing of N-linked Oligosaccharides Directly from Protein Gels: In-Gel Deglycosylation Followed by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry and Normal-Phase High-Performance Liquid Chromatography." *Analytical Biochemistry*, 250, 82-101.
- Laemmli, U. K. (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." *Nature*, 227(259), 680-5.
- Lanahan, A., and Worley, P. (1998). "Immediate-Early Genes and Synaptic Function." *Neurobiol Learn Mem*, 70(1/2), 37-43.
- Lander, C., Kind, P., Maleski, M., and Hockfield, S. (1997). "A family of activity-dependent neuronal cell-surface chondroitin sulfate proteoglycans in cat visual cortex." *Journal of Neuroscience*, 17(6), 1928-1939.

- Lauffenburger, D. A., and Horwitz, A. F. (1996). "Cell-Migration - a Physically Integrated Molecular Process." *Cell*, 84(3), 359-369.
- Lehmann, J. (1998). *Carbohydrates, structure and biology*, Haines, H.A., translator, Georg Thieme Verlag, Thieme.
- Lennon, K., Bird, A., Chen, Y. F., Pretel, R., and Kukuruzinska, M. A. (1997). "The dual role of mRNA half-lives in the expression of the yeast ALG7 gene." *Molecular and Cellular Biochemistry*, 169(1-2), 95-106.
- Likhoshervostov, L. M., Novikova, O. S., Derevitskaya, V. A., and Kochetkov, N. K. (1990). "A Selective Method For Sequential Splitting of O-Linked and N-Linked Glycans From N,O-Glycoproteins." *Carbohydrate Research*, 199(1), 67-76.
- Luthi, A., Laurent, J. P., Figurov, A., Muller, D., and Schachner, M. (1994). "Hippocampal long-term potentiation and neural cell adhesion molecules L1 and NCAM." *Nature*, 372(6508), 777-9.
- Ma, J. Y., and Colley, K. J. (1996). "A Disulfide-Bonded Dimer of the Golgi Beta-Galactoside Alpha-2,6- Sialyltransferase Is Catalytically Inactive Yet Still Retains the Ability to Bind Galactose." *Journal of Biological Chemistry*, 271(13), 7758-7766.
- Maguire, T. M., Thakore, J., Dinan, T. G., Hopwood, S., and Breen, K. C. (1997). "Plasma sialyltransferase levels in psychiatric disorders as a possible indicator of HPA axis function." *Biological Psychiatry*, 41(11), 1131-1136.
- Mai, J. K., Andressen, C., and Ashwell, K. W. S. (1998). "Demarcation of prosencephalic regions by CD15-positive radial glia." *European Journal Of Neuroscience*, 10(2), 746-751.
- Mandelkow, E., and Mandelkow, E. M. (1994). "Microtubule Structure." *Current Opinion in Structural Biology*, 4(2), 171-179.
- Mandelkow, E. M., and Mandelkow, E. (1993). "Tau as a Marker For Alzheimers-Disease." *Trends in Biochemical Sciences*, 18(12), 480-483.
- Margolis, R. U., and Margolis, R. K. (1977). "Metabolism and function of glycoproteins and glycosaminoglycans in nervous tissue." *Int. J. Biochem.*, 8, 85-91.
- Margolis, R. U., and Margolis, R. K. (1989). *Neurobiology of Glycoconjugates*, Plenum Press, New York.
- Mayanil, C. S. K., and Knepper, P. A. (1993). "Synaptic Vesicle and Synaptic Membrane-Glycoproteins During Prenatal- Development and Postnatal-Development of Mouse Cerebral-Cortex, Cerebellum and Spinal-Cord." *Developmental Neuroscience*, 15(2), 133-145.
- McCabe, N. R., and Rose, S. P. R. (1985). "Passive-Avoidance Training Increases Fucose Incorporation into Glycoproteins in Chick Forebrain Slices In Vitro." *Neurochemical Research*, 10(8), 1083-1095.
- McEwen, B. S., and Sapolsky, R. M. (1995). "Stress and cognitive function." *Curr Opin Neurobiol*, 5(2), 205-16.
- Medina, J. H., and Izquierdo, I. (1995). "Retrograde messengers, long-term potentiation and memory." *Brain Research Reviews*, 21, 185-194.
- Meresse, S., Bauer, U., Ludwig, T., Mauxion, F., Schmidt, A., and Hoflack, B. (1993). "Molecular-Basis of Transport to Lysosomes." *M S-Medecine Sciences*, 9(2), 148-156.
- Mollicone, R., Candelier, J. J., Mennesson, B., Couillin, P., Venot, A. P., and Oriol, R. (1992). "Five specificity patterns of (1----3)-alpha-L-fucosyltransferase activity defined by use of synthetic oligosaccharide acceptors. Differential expression of the enzymes during human embryonic development and in adult tissues." *Carbohydr Res*, 228(1), 265-76.

- Morgan, D. G., and Routtenberg, A. (1979). "The incorporation of intrastrially injected [3H]fucose into electrophoretically separated synaptosomal glycoproteins. II. The influence of passive avoidance training." *Brain Res*, 179(2), 343-54.
- Muhlenhoff, M., Eckhardt, M., Bethé, A., Frosch, M., and Gerardyschahn, R. (1996). "Polysialylation of NCAM By a Single Enzyme." *Current Biology*, 6(9), 1188-1191.
- Muller, D., Wang, C., Skibo, G., Toni, N., Cremer, H., Calaora, V., Rougon, G., and Kiss, J. Z. (1996). "PSA-NCAM Is Required For Activity-Induced Synaptic Plasticity." *Neuron*, 17(3), 413-422.
- Nair, S. M., Zhao, Z., Chou, D. K. H., Tobet, S. A., and Jungalwala, F. B. (1998). "Expression of HNK-1 carbohydrate and its binding protein, SBP-1, in apposing cell surfaces in cerebral cortex and cerebellum." *Neuroscience*, 85(3), 759-771.
- Nayak, A., Zastrow, D. J., Lickteig, R., Zahniser, N. R., and Browning, M. D. (1998). "Maintenance of late-phase LTP is accompanied by PKA-dependent increase in AMPA receptor synthesis." *Nature*, 394(6694), 680-3.
- Neue, U. D., and Phoebe, C. H. (1997). "High-performance Liquid Chromatography of Derivatized and Non-Derivatized Oligosaccharides: A Review." *A Laboratory Guide to Glycoconjugate Analysis*, Birkhauser Verlag, Basel, 1-22.
- Nilsson, T., and Warren, G. (1994). "Retention and retrieval in the endoplasmic reticulum and the Golgi apparatus." *Curr Opin Cell Biol*, 6(4), 517-21.
- Nishimura, H., Kawabata, S., Kisiel, W., Hase, S., Ikenaka, T., Takao, T., Shimonishi, Y., and Iwanaga, S. (1989). "Identification of a Disaccharide (Xyl-Glc) and a Trisaccharide (Xyl2-Glc) O-Glycosidically Linked to a Serine Residue in the 1st Epidermal Growth Factor-Like Domain of Human Factor-VII and Factor-IX and Protein-Z and Bovine Protein-Z." *Journal of Biological Chemistry*, 264(34), 20320-20325.
- Olsen, M., Krog, E., and Bock, E. (1993). "Glycosylation of NCAM: A member of the immunoglobulin superfamily." *Complex Carbohydrates in Drug Research*, K. Bock and H. Clausen, eds., Munksgaard, Copenhagen, 316-336.
- O'Neill, R. A. (1996). "Enzymatic release of oligosaccharides from glycoproteins for chromatographic and electrophoretic analysis." *Journal of Chromatography*, 720, 201-215.
- Palestini, P., Toppi, N., Ferraretto, A., Pitto, M., and Masserini, M. (1997). "Ganglioside lateralization in the brain of female rats." *Journal of Neuroscience Research*, 50(4), 643-648.
- Parker, C. G., Fessler, L. I., Nelson, R. E., and Fessler, J. H. (1995). "UDP-Glucose - Glycoprotein Glucosyltransferase as a Folding Sensor in the Endoplasmic-Reticulum." *Trends in Glycoscience and Glycotechnology*, 7(37), 451-452.
- Patel, T., Bruce, J., Merry, A., Bigge, C., Wormald, M., Jaques, A., and Parekh, R. (1993). "Use of hydrazine to release in intact and unreduced form both N- and O-linked oligosaccharides from glycoproteins." *Biochemistry*, 32(2), 659-693.
- Pereira, M. E. A., Kisailus, E.C., Gruezo, F., Kabat, E.A. (1978). "Immunochemical studies on the combining site of the blood group H-specific lectin from *Ulex europaeus* seeds." *Arch. Biochem. Biophys.*, 185, 108-115.
- Perez, S., Mouhous-Riou, N., Nifantev, N. E., Tsvetkov, Y. E., Bachet, B., and Imberty, A. (1996). "Crystal and molecular structure of a histo-blood group antigen involved in cell adhesion: the Lewis x trisaccharide." *Glycobiology*, 6(5), 537-542.
- Pestean, A., Krizbai, I., Bottcher, H., Parducz, A., Joo, F., and Wolff, J. R. (1995). "Identification of the *Ulex-Europaeus* Agglutinin-I-Binding Protein as a Unique Glycoform of the Neural Cell-Adhesion Molecule in the Olfactory Sensory Axons of Adult-Rats." *Neuroscience Letters*, 195(2), 117-120.

- Pierce, M., Buckhaults, P., Chen, L., and Fregien, N. (1997). "Regulation of N-acetylglucosaminyltransferase V and Asn-linked oligosaccharide beta(1,6) branching by a growth factor signaling pathway and effects on cell adhesion and metastatic potential." *Glycoconjugate Journal*, 14(5), 623-630.
- Piller, V., Meunier, D., Bureau, N., Piller, F., (1997). "The role of N-glycans in the intracellular routing and activity of a glycosyltransferase." *Glycoconjugate Journal*, 14 (supplement 1), S40.
- Pizzorusso, T., Fagiolini, M., Porciatti, V., and Maffei, L. (1997). "Temporal aspects of contrast visual evoked potentials in the pigmented rat: Effect of dark rearing." *Vision Research*, 37(4), 389-395.
- Plummer, T. H., Elder, J. H., Alexander, S., Phelan, A. W., and Tarentino, A. L. (1984). "Demonstration of Peptide-N-Glycosidase-F Activity in Endo-Beta-N-Acetylglucosaminidase F Preparations." *Journal of Biological Chemistry*, 259(17), 700-704.
- Pohle, W., Ruthrich, H. L., Popov, N., and Matthies, H. (1979). "Fucose incorporation into rat hippocampus structures after acquisition of a brightness discrimination. A histoautoradiographic analysis." *Acta Biol Med Ger*, 38(1), 53-63.
- Popov, N., Ruthrich, H. L., Pohle, W., Schulzeck, S., Matthies, H., Damstra, T., Entingh, D., Wilson, J. E., Glassman, E., Routenberg, A., George, K. D., Davis, L. G., Brunngraber, E. G., Damstra-Entingh, T., Entingh, D., Wilson, J. E., Glassman, E., Koenig, H., Di Giamberardino, L., and Bennett, G. (1976). "Increased fucose incorporation into rat hippocampus during learning. A biochemical and microautoradiographic study." *Brain Res*, 101(2), 295-304.
- Popov, N., Schulzeck, S., Pohle, W., and Matthies, H. (1980). "Changes in the incorporation of [3H]fucose into rat hippocampus after acquisition of a brightness discrimination reaction. An electrophoretic study." *Neuroscience*, 5(1), 161-7.
- Preuss, T. M. (1995). "The Argument from Animals to Humans in Cognitive Neuroscience." *The Cognitive Neurosciences*, MIT Press, London, 1227-1241.
- Priatel, J. J., Sarkar, M., Schächter, H., and Marth, J. D. (1997). "Isolation, characterization and inactivation of the mouse Mgat3 gene: the bisecting N-acetylglucosamine in asparagine-linked oligosaccharides appears dispensable for viability and reproduction." *Glycobiology*, 7(1), 45-56.
- Prime, S. B., Shipston, N. F., and Merry, T. H. (1997). "Enzymatic Sequence Analysis of Glycoprotein Glycans." *A Laboratory Guide to Glycoconjugate Analysis*, Birkhauser Verlag, Basel, 235-260.
- Qu, B. X., Ziak, M., Zuber, C., and Roth, J. (1996). "Poly(Alpha-2,8-Deaminoneuraminic Acid) Is Expressed in Lung On a Single 150-kDa Glycoprotein and Is an Oncodevelopmental Antigen." *Proceedings of the National Academy of Sciences of the United States of America*, 93(17), 8995-8998.
- Ratvala, H., and Pihlaskari, R. (1987). "Isolation and Some Characteristics of an Adhesive Factor of Brain That Enhances Neurite Outgrowth in Central Neurons." *Journal of Biological Chemistry*, 262(34), 16625-16635.
- Reid, S. N. M., and Daw, N. W. (1995). "Dark-Rearing Changes Dendritic Microtubule-Associated Protein-2 (MAP2) But Not Subplate Neurons in Cat Visual-Cortex." *Journal of Comparative Neurology*, 359(1), 38-47.
- Reuter, G., Schauer, R. (1994). "Determination of Sialic Acids." *Guides to Techniques in Glycobiology*, W. J. Lennarz, Hart, G.W., ed., Academic Press, San Diego, 168-199.
- Rickard, N. S., Poot, A. C., Gibbs, M. E., and Ng, K. T. (1994). "Both non-NMDA and NMDA glutamate receptors are necessary for memory consolidation in the day-old chick." *Behav Neural Biol*, 62(1), 33-40.

- Roberts, C., Platt, N., Streit, A., Schachner, M., and Stern, C. D. (1991). "The L5 epitope: an early marker for neural induction in the chick embryo and its involvement in inductive interactions." *Development*, 112, 959-970.
- Rogers, L. J. (1995). *The development of brain and behaviour in the chicken*, CAB International.
- Rose, S. P. R. (1981). "What Should a Biochemistry of Learning and Memory Be About." *Neuroscience*, 6(5), 811-821.
- Rose, S. P. R. (1991). "How Chicks Make Memories - the Cellular Cascade From C-Fos to Dendritic Remodeling." *Trends in Neurosciences*, 14(9), 390-397.
- Rose, S. P. R. (1996). "Cell adhesion molecules and the transition from short- to long-term memory." *Journal of Physiology-Paris*, 90(5-6), 387-391.
- Rose, S. P. R., and Csillag, A. (1985). "Passive-Avoidance Training Results in Lasting Changes in Deoxyglucose Metabolism in Left-Hemisphere Regions of Chick Brain." *Behavioral and Neural Biology*, 44(2), 315-324.
- Rose, S. P. R., and Harding, S. (1984). "Training Increases [Fucose-H-3 Incorporation in Chick Brain Only If Followed By Memory Storage." *Neuroscience*, 12(2), 663-667.
- Routtenberg, A., George, K. D., Davis, L. G., and Brunngraber, E. G. (1974). "Memory consolidation and fucosylation of crude synaptosomal glycoproteins resolved by gel electrophoresis: a regional study." *Behav Biol*, 12(4), 461-75.
- Rozenzweig, M. R. (1996). "Aspects of the search for neural mechanisms of memory." *Ann. Rev. Psychol.*, 47, 1-32.
- Rudd, P. M., Guile, G. R., Kuster, B., Harvey, D. J., Opdenakker, G., and Dwek, R. A. (1997). "Oligosaccharide sequencing technology." *Nature*, 388(6638), 205-207.
- Rudd, P. M. and Dwek R.A.(1997). "Glycosylation: Heterogeneity and the 3D Structure of Proteins." *Critical Reviews in Biochemistry and Molecular Biology*, 32(1), 1-100.
- Rusakov, D. A., Stewart, M. G., Davies, H. A., and Harrison, E. (1993). "Spatial Re-Arrangement of the Vesicle Apparatus in Forebrain Synapses of Chicks 30 Min After Passive-Avoidance Training." *Neuroscience Letters*, 154(1-2), 13-16.
- Rutishauser, U., and Landmesser, L. (1996). "Polysialic Acid in the Vertebrate Nervous-System - a Promoter of Plasticity in Cell-Cell Interactions." *Trends in Neurosciences*, 19(10), 422-427.
- Salin, P. A., Malenka, R. C., and Nicoll, R. A. (1996). "Cyclic AMP mediates a presynaptic form of LTP at cerebellar parallel fiber synapses." *Neuron*, 16(4), 797-803.
- Salinska, E. J., Chaudhury, D., Bourne, R. C., and Rose, S. P. R. (1998). "Passive avoidance training results in changes in intracellular calcium concentration following stimulation of voltage- and ligand-gated calcium channels." *Submitted*.
- Sandi, C., and Rose, S. P. R. (1994a). "Corticosteroid Receptor Antagonists Are Amnesic For Passive- Avoidance Learning in Day-Old Chicks." *European Journal of Neuroscience*, 6(8), 1292-1297.
- Sandi, C., and Rose, S. P. R. (1994b). "Corticosterone Enhances Long-Term Retention in One-Day-Old Chicks Trained in a Weak Passive-Avoidance Learning-Paradigm." *Brain Research*, 647(1), 106-112.
- Sandi, C., and Rose, S. P. R. (1997). "Protein synthesis- and fucosylation-dependent mechanisms in corticosterone facilitation of long-term memory in the chick." *Behavioral Neuroscience*, 111(5), 1098-1104.
- Satoh, J., and Kim, S. U. (1994). "Differential Expression of LewisX and Sialyl-LewisX Antigens in Fetal Human Neural Cells in Culture." *J. Neurosci. Res.*, 37, 466-474.

- Schauer, R., Kelm, S., Reuter, G., Roggentin, P., and Shaw, L. (1995). "Biochemistry and Role of Sialic acids." *Biology of the Sialic Acids*, A. Rosenberg, ed., Plenum Press, New York, 7-67.
- Schellfefe, P., Peranen, J., Simons, K. (1995). "N-glycans as apical sorting signals in epithelial cells." *Nature*, 378, 96-98.
- Schmidt, R. (1995). "Cell-adhesion molecules in memory formation." *Behav Brain Res*, 66(1-2), 65-72.
- Schmitz, B., Peter-Katalinic, J., Egge, H., Schachner, M. (1993). "Monoclonal antibodies raised against membrane glycoproteins from mouse brain recognize N-linked oligomannosidic glycans." *Glycobiology*, 3(9), 609-617.
- Schnaar, R. L., Collins, B. E., Wright, L. P., Kiso, M., Tropak, M. B., Roder, J. C., and Crocker, P. R. (1998). "Myelin-associated glycoprotein binding to gangliosides - Structural specificity and functional implications." *Annals of the New York Academy of Sciences*, 845, 92-105.
- Scholey, A. B., Mileusnic, R., Schachner, M., and Rose, S. P. R. (1995). "A Role for a Chicken Homologue of the Neural Cell Adhesion Molecule L1 in Consolidation of Memory for a Passive Avoidance Task in the Chick." *Learning and Memory*, 2, 17-25.
- Scholey, A. B., Rose, S. P. R., Zamani, M. R., Bock, E., and Schachner, M. (1993). "A Role For the Neural Cell-Adhesion Molecule in a Late, Consolidating Phase of Glycoprotein-Synthesis 6 Hours Following Passive-Avoidance Training of the Young Chick." *Neuroscience*, 55(2), 499-509.
- Schoups, A. A., Elliott, R. C., Friedman, W. J., and Black, I. B. (1995). "NGF and BDNF Are Differentially Modulated By Visual Experience in the Developing Geniculocortical Pathway." *Developmental Brain Research*, 86(1-2), 326-334.
- Schwartz, M. A., Schaller, M. D., and Ginsberg, M. H. (1995). "Integrins: Emerging paradigms of Signal Transduction." *Annu. Rev. Dev. Biol.*, 11, 549-599.
- Seki, T., and Arai, Y. (1991). "The Persistent Expression of a Highly Polysialylated NCAM in the Dentate Gyrus of the Adult-Rat." *Neuroscience Research*, 12(4), 503-513.
- Seki, T., and Arai, Y. (1993). "Highly Polysialylated Neural Cell-Adhesion Molecule (NCAM-H) Is Expressed By Newly Generated Granule Cells in the Dentate Gyrus of the Adult-Rat." *Journal of Neuroscience*, 13(6), 2351-2358.
- Seki, T., and Rutishauser, U. (1998). "Removal of polysialic acid neural cell adhesion molecule induces aberrant mossy fiber innervation and ectopic synaptogenesis in the hippocampus." *Journal of Neuroscience*, 18(10), 3757-3766.
- Shakineshleman, S. H. (1996). "Regulation of N-Linked Core-Glycosylation." *Trends In Glycoscience and Glycotechnology*, 8(40), 115-130.
- Shashoua, V. E. (1991). "Ependymin, a brain extracellular glycoprotein, and CNS plasticity." *Ann N Y Acad Sci*, 627, 94-114.
- Shimizu, H., Ochiai, K., Ikenaka, K., Mikoshiba, K., and Hase, S. (1993). "Structures of N-Linked Sugar Chains Expressed Mainly in Mouse-Brain." *Journal of Biochemistry*, 114(3), 334-338.
- Silva, A. J., Kogan, J. H., Frankland, P. W., and Kida, S. (1998). "CREB and memory." *Annual Review of Neuroscience*, 21, 127-148.
- Smalla, K.-H., Angenstein, F., Richter, K., Gundelfinger, E. D., and Staak, S. (1998). "Identification of fucose alpha(1-2) galactose epitope-containing glycoproteins from rat hippocampus." *Neuroreport*, 9, 813-817.
- Smith, M. A., Makino, S., Kvetnansky, R., and Post, R. M. (1995). "Stress and Glucocorticoids Affect the Expression of Brain-Derived Neurotrophic Factor and

- Neurotrophin-3 Messenger-RNAs in the Hippocampus.” *Journal of Neuroscience*, 15(3 Pt1), 1768-1777.
- Sokoloff, L., Reivich, M., Kennedy, C., Des Rosiers, M. H., Patlak, C. S., Pettigrew, K. D., Sakurada, O., and Shinohara, M. (1977). “The [¹⁴C]deoxyglucose method for the measurement of local cerebral glucose utilization: theory, procedure, and normal values in the conscious and anesthetized albino rat.” *J Neurochem*, 28(5), 897-916.
- Song, J., Zipse, B. (1995). “Targeting of the Neuronal Subsets Mediated by their Sequentially Expressed Carbohydrate Markers.” *Neuron*, 14, 537-547.
- Spik, G. (1995). “Methods on Glycoconjugates a laboratory manual.”, A. Verbert, ed., Harwood Academic Publishers, Chur, 27-31.
- Squire, L. R. (1992). “Memory in the hippocampus: A synthesis from findings with rats, monkeys, and humans.” *Psychol. Review*, 99, 195-231.
- Squires, L. R., and Barondes, S. H. (1972). “Variable decay of memory and its recovery in cycloheximide-treated mice.” *Proc. Natl. Acad. Sci.*, 69, 1416-1420.
- Stahn, R., Schafer, H., Kernchen, F., and Schreiber, J. (1998). “Multivalent sialyl Lewis x ligands of definite structures as inhibitors of E-selectin mediated cell adhesion.” *Glycobiology*, 8(4), 311-319.
- Stanton, P. K., and Sarvey, J. M. (1984). “Blockade of long-term potentiation in rat hippocampal CA1 region by inhibitors of protein synthesis.” *J Neurosci*, 4(12), 3080-8.
- Stewart, M. G., Bourne, R. C., and Steele, R. J. (1992). “Quantitative Autoradiographic Demonstration of Changes in Binding to NMDA-Sensitive [³H] Glutamate and [³H]MK801, But Not [³H]AMPA Receptors in Chick Forebrain 30-Min After Passive-Avoidance Training.” *European Journal of Neuroscience*, 4(10), 936-943.
- Stewart, M. G., Csillag, A., and Rose, S. P. R. (1987). “Alterations in Synaptic Structure in the Paleostriatal Complex of the Domestic Chick, *Gallus-Domesticus*, Following Passive-Avoidance Training.” *Brain Research*, 426(1), 69-81.
- Stewart, M. G., Rose, S. P. R., King, T. S., Gabbott, P. L. A., and Bourne, R. (1984). “Hemispheric-Asymmetry of Synapses in Chick Medial Hyperstriatum Ventrale Following Passive-Avoidance Training - a Stereological Investigation.” *Developmental Brain Research*, 12(2), 261-269.
- Storms, S. D., Kim, A. C., Tran, B. H. T., Cole, G. J., and Murray, B. A. (1996). “NCAM-Mediated Adhesion Of Transfected Cells to Agrin.” *Cell Adhesion and Communication*, 3(6), 497-509.
- Streit, A., Yuen, C. T., Loveless, W. R., Lawson, A. M., Fine, J., Scmitz, B., Feizi, T., and Stern, C. D. (1996). “The LewX Carbohydrate Sequence Is Recognized by Antibody to L5, a Functional Antigen in Early Neural Development.” *Journal of Neurochemistry*, 66(2), 834-844.
- Stubbs, H. J., Lih, J. J., Gustafson, T. L., and Rice, K. G. (1996). “Influence of core fucosylation on the flexibility of a biantennary N- linked oligosaccharide.” *Biochemistry*, 35(3), 937-47.
- Sui, N., and Rose, S. P. R. (1997). “Effects of dark rearing and light exposure on memory for a passive avoidance task in day-old chicks.” *Neurobiology of Learning and Memory*, 68(3), 230-238.
- Sui, N., Sandi, C., and Rose, S. P. R. (1997). “Interactions of corticosterone and embryonic light deprivation on memory retention in day-old chicks.” *Developmental Brain Research*, 101(1-2), 269-272.
- Sukumar, R., Rose, S. P., and Burgoyne, R. D. (1980). “Increased incorporation of [³H]fucose into chick brain glycoproteins following training on a passive avoidance task.” *J Neurochem*, 34(4), 1000-6.

- Sultan, A. S., Miyoshi, E., Ihara, Y., Nishikawa, A., Tsukada, Y., and Taniguchi, N. (1997). "Bisecting GlcNAc structures act as negative sorting signals for cell surface glycoproteins in forskolin-treated rat hepatoma cells." *Journal of Biological Chemistry*, 272(5), 2866-2872.
- Tamkun, M. M., and Fambrough, D. M. (1986). "The (Na⁺ + K⁺)-ATPase of chick sensory neurons. Studies on biosynthesis and intracellular transport." *J Biol Chem*, 261(3), 1009-19.
- Tang, L. X., Hung, C. P., and Schuman, E. M. (1998). "A role for the cadherin family of cell adhesion molecules in hippocampal long term potentiation." *Neuron*, 20(6), 1165-1175.
- Taniguchi, N., and Ihara, Y. (1995). "Recent progress in the molecular biology of the cloned N-acetylglucosaminyltransferases." *Glycoconjugate Journal*, 12, 733-738.
- Terayama, K., Oka, S., Seiki, T., Miki, Y., Nakamura, A., Kozutsumi, Y., Takio, K., and Kawasaki, T. (1997). "Cloning and functional expression of a novel glucuronyltransferase involved in the biosynthesis of the carbohydrate epitope HNK-1." *Proceedings of the National Academy of Sciences of the United States of America*, 94(12), 6093-6098.
- Thoenen, H. (1995). "The changing scene of neurotrophic factors." *Trends in Neurosciences*, 14, 165-170.
- Thomas, D., Lehmann, S., Kuchler, S., Marschal, P., and ZAnetta, J. P. (1994). "Differential expression of an endogenous mannose-binding protein R1 during muscle development and regeneration delineating its role in myoblast fusion." *Glycobiology*, 4, 23-28.
- Torre, E. R., and Steward, O. (1996). "Protein-Synthesis Within Dendrites - Glycosylation of Newly Synthesized Proteins in Dendrites of Hippocampal-Neurons in Culture." *Journal of Neuroscience*, 16(19), 5967-5978.
- Varki, A. (1997). "Sialic acids as ligands in recognition phenomena." *Faseb Journal*, 11(4), 248-255.
- Varki, A., and Freeze, H. H. (1994). "The major Glycosylation Pathways of Mammalian Membranes - A Summary." *Subcellular Biochemistry*, A. H. H. Maddy, J. R., ed., Plenum Press, New York, 71-100.
- Verbert, A. (1995). *Methods on Glycoconjugates a laboratory manual*, Harwood Academic Publishers, Chur.
- Vlasova, E. V., Lukin, S. V., Khaidukov, S. V., Astapova, M. V., and Bovin, N. V. (1996). "Fine Carbohydrate Specificity Of Anti-CD15 and Anti-CD15s Monoclonal-Antibodies Submitted to the Adhesion Structures Panel." *Tissue Antigens*, 48(4-II), AS208-AS208.
- Voshol, H., van Zuylen, W. E. M., Orberger, G., and Vliegenthart, J. F. G. (1996). "Structure of the HNK-1 Carbohydrate Epitope on Bovine Peripheral Myelin Glycoprotein P0." *J. Biol. Chem.*, 271(38), 22957-22960.
- Wang, Y., Lee, G. F., Kelley, R. F., and Spellman, M. W. (1996). "Identification of a GDP-L-fucose:polypeptide fucosyltransferase and enzymatic addition of O-linked fucose to EGF domains." *Glycobiology*, 6(8), 837-842.
- Wartiovaara, J. J., Pihlaskari, R., and Rauvala, H. (1990). "Immunogold Localization of a Novel Adhesive Molecule, Amphoterin (P- 30) in Rotary-Shadowed Human Fibroblasts." *Institute of Physics Conference Series*(98), 685-686.
- Welzl, H., Becker, C. G., Artola, A., and Schachner, M. (1996). "Polysialic Acid (PSA) Associated with the Neural Cell-Adhesion Molecule (NCAM) May Play a Role in Spatial-Learning and LTP in Rats." *Journal of Neurochemistry*, 66(S2), S 17-S 17.

- Wing, D. R., Rademacher, T.W., Schmitz, B., Schachner, M., Dwek, R.A. (1992). "Comparative glycosylation in neural adhesion molecules." *Bioch. Soc. Trans.*, 20, 386-390.
- Woolf, N. J. (1998). "A structural basis for memory storage in mammals." *Prog Neurobiol*, 55(1), 59-77.
- Xiao, P., Bahr, B. A., Staubli, U., Vanderklisch, P. W., and Lynch, G. (1991). "Evidence that matrix recognition contributes to stabilization but not induction of LTP [published erratum appears in *Neuroreport* 1991 Sep;2(9):following 546]." *Neuroreport*, 2(8), 461-4.
- Yan, L., Wilkins, P. P., Alvarez-Manilla, G., Do, S. I., Smith, D. F., and Cummings, R. D. (1997). "Immobilized Lotus tetragonolobus agglutinin binds oligosaccharides containing the Le(x) determinant." *Glycoconj J*, 14(1), 45-55.
- Yao, P. J., and Coleman, P. D. (1998). "Reduction of O-linked N-acetylglucosamine-modified assembly protein-3 in Alzheimer's disease." *Journal of Neuroscience*, 18(7), 2399-2411.
- Zambenedetti, P., Giordano, R., and Zatta, P. (1996). "Identification of Lectin-Binding Sites in the Rat-Brain." *Glycoconjugate Journal*, 13(3), 341-346.
- Zanetta, J.-P., Reeber, A., Vincendon, G., Gombos, G. (1977). "Synaptosomal Plasma Membrane Glycoproteins. II. Isolation of Fucosyl-Glycoproteins by Affinity Chromatography on the Ulex Europaeus Lectin Specific for L-Fucose." *Brain Research*, 138, 317-328.
- Zanetta, J. P., Dontenwill, M., Meyer, A., and Roussel, G. (1985). "Isolation and Immunohistochemical Localization of a Lectin-like Molecule from the Rat Cerebellum." *Dev. Brain Res.*, 17, 233-243.
- Zhao, W., Ng, K. T., and Sedman, G. L. (1995a). "Passive avoidance learning induced change in GAP43 phosphorylation in day-old chicks." *Brain Res Bull*, 36(1), 11-7.
- Zhao, W. Q., Polya, G. M., Wang, B. H., Gibbs, M. E., Sedman, G. L., and Ng, K. T. (1995b). "Inhibitors of cAMP-dependent protein kinase impair long-term memory formation in day-old chicks." *Neurobiol Learn Mem*, 64(2), 106-18.
- Ziak, M., Kerjaschki, D., Farquhar, M. G., and Roth, J. (1996). "The single poly alpha 2,8 deaminoneuraminic acid bearing glycoprotein in rat kidney corresponds to megalin." *Molecular Biology of the Cell*, 7(SS), 3466-3466.
- Zipser, B., and Cole, R. N. (1991). "A Mannose-specific Recognition Mediates the Defasciculation of Axons in the Leech CNS." *J. Neurosci.*, 11, 3471-3480.
- Zuber, C., Lackie, P. M., Catterall, W. A., and Roth, J. (1992). "Polysialic Acid Is Associated With Sodium-Channels and the Neural Cell-Adhesion Molecule N-Cam in Adult-Rat Brain." *Journal of Biological Chemistry*, 267(14), 9965-9971.